Mark H. Tuszynski *Editor*

Translational Neuroscience

Fundamental Approaches for Neurological Disorders

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Chapter 1
Introduction

Mark H. Tuszynski

Keywords Transcriptome • Proteome • Gene therapy • Anti-sense therapy • Alzheimer's disease • Parkinson's disease • Autism • ALS

Introduction

The last 20–30 years have brought about a revolution in the technology with which we study the nervous system and with it the potential to translate new therapies to humans.

First and perhaps foremost, tools of genomics have transformed our ability to understand genetic mechanisms of neural function and dysfunction. These tools include whole genome sequencing, RNA sequencing, interrogation of epigenetic mechanisms, and new understanding of the complex roles that RNA species, such as microRNAs, play in regulating cell function. The capacity to interrogate these genetic mechanisms—the "transcriptome"—has required the development of computational tools to manage enormous amounts of data [1–4]. But these data are bringing about a massive transformation in our understanding of the nervous system, individual susceptibility to disease, and treatment.

Together with advances in the understanding of genetic control of cell function have come increasingly sophisticated tools for manipulating genes. These range from recent advances in transgenic mouse models, such as bacterial artificial chromosome (BAC) models [5], to precise gene editing tools such as CRISPR/Cas9 technology [6]. The latter can be applied to cells in culture or to germ cell lines to rapidly create and screen transgenic mouse models. Indeed, rapid genetic and mutant screens in nonmammalian species, such as drosophila [7] and *C. elegans* [8], accelerated the search for genes related to disease mechanism and the screening of potential treatments [9]. With these capabilities comes the hypothetical capacity to edit *human* germ lines, a possibility that is the subject of intense ethical scrutiny [10].

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Together with tools to manipulate the genome have come tools to suppress gene function, including anti-sense therapies and vector-mediated knockdown of gene function [11, 12], and gene editing [13].

Moving to the next level of cellular control of function, tools for probing the protein compartment of the cell (the proteome) have advanced in both speed and sensitivity, to the point that we can understand the impact of specific manipulations on vast sets of cellular proteins and phosphoprotein species with unprecedented sensitivity and efficiency [14, 15]. Other cellular compartments can also be probed as never before, including the lipidome [16] and metabolome [17]. Indeed, even the microbiome (e.g., the influence of gut flora) has been identified as a potentially important modulator of disease mechanisms in the CNS [18, 19].

Our ability to record and stimulate neural circuits based on advances in neuroengineering and bioengineering has yielded novel insights into the fundamental nature of neural circuit assembly during the course of normal learning and memory [20] and in the setting of dysfunction associated with neurodegenerative disorders or such diseases as epilepsy [21]. A new generation of therapies has emerged from this work, from chronically implanted stimulators for Parkinson's disease [22] and dystonia [23] to brain–machine interface after nervous system injury [24, 25].

Imaging has also advanced dramatically, from the level of novel microscopes that can image the single cells and subcellular structures repeatedly over time periods of months [26, 27] to whole-brain quantitative MRI [28]. Novel PET scan markers such as PIB imaging [29] and, potentially, tau imaging [30] in Alzheimer's disease are enabling the clinical testing of potential therapies even *before* symptom onset [31]. Structural MRI may emerge as a biomarker of disease progression and response to therapy [28]. Functional MRI of the actively behaving brain has provided a wealth of information regarding the role of brain regions in behavior [32], which in turn may contribute to the identification of mechanisms of disease [33], and from there to therapies. Several of the preceding advances have been utilized to explore the existence and utility of biomarkers for better disease diagnosis and monitors of treatment in blood and CSF [34].

Neural stem cell technologies have advanced to a breathtaking degree in the last 20 years, from the initial discovery that self-renewing cells exist in all the adult mammalian nervous system [35, 36] to the current ability to generate an individual adult's own stem cells and their derivatives using specified sets of transcription factors [37, 38]. Indeed, the technology for directly generating neural stem cells and specific neural cells from "terminally" differentiated adult cells has rapidly advanced to neural transdifferentiation, in which even the induced pluripotent stem cell stages of cells can be bypassed [39]. Initial therapeutic efforts in humans have begun with neural stem cells [40].

Each of these technologies has been used in an effort to gain new insights into mechanisms underlying human disease and to generate novel therapies. For this purpose, screening tools are frequently used, which have grown in sophistication and capacity, ranging from high-throughput in vitro screens capable of screening hundreds of thousands of compounds that engage an in vitro readout [9] to traditional in vivo animal models of disease that often plod along in much the traditional manner of the

last century [41]. Yet, at the end of that chain of screens—the in vivo animal model of disease—lies the final and most important method for assessing translational therapies that might move to the clinic.

In short, this is an astonishing era of discovery. But what have been the tangible advances in diagnosis and treatment that we have achieved in translational neuroscience up to this point? What has the investment of several hundred billions of dollars, euros, pounds, yen, and renminbi yielded in understanding human disease mechanism and creating novel and more effective treatments?

Progress has begun to occur. These are a few examples:.

1. Genetic analyses of the last 20 years, accelerating markedly in the last few years, have enabled the discovery of the basis of several human diseases, including spinal muscular atrophy, spinocerebellar ataxia, system degenerations such as progressive supranuclear palsy, inherited forms of Alzheimer's disease and Parkinson's disease, and some forms of childhood epilepsy and autism. The discovery of mutations and small nucleotide polymorphisms associated with disease risk has in turn yielded insights into disease mechanism. For example, alternative splicing in some forms of human spinal muscular atrophy has been shown to result in reduced levels of SMN protein, and chemical screening and optimization of small compounds have identified new drug candidates that are beginning clinical trials in this untreatable and tragic disorder [9]. In autism spectrum disorder, genetic screening has led to the identification of a metabolic deficiency that may be treatable with dietary modification [42].
2. Genetic analyses have also recently identified distinct subtypes of primary nervous system tumors that were previously indistinguishable on the basis of pure anatomical/pathological analysis. For example, genetic hetereogeneity in types of grade III astrocytomas now can be associated with distinct differences in prognosis that direct the selection of unique therapies and can make a difference of literally years in survival for individual patients [43]. This is the beginning of a major advance in the treatment of primary nervous system malignancy.
3. Advances in monoclonal antibody therapy have led in the last decade to more effective, if more risky, therapies for neuroimmune disorders. For example, a monoclonal antibody targeted toward integrin receptors significantly reduces disease progression in multiple sclerosis, albeit with a risk of opportunistic infection [44, 45]. The list of potential disease indications for directed immunotherapy is expanding rapidly, including active efforts in Alzheimer's disease that target both beta amyloid and tau and efforts to target alpha synuclein in Parkinson's disease.
4. The field of gene therapy has progressed in the last few years. Clinical trials in Alzheimer's disease, Parkinson's disease, epilepsy, pain, and congenital eye disease have progressed to the clinic, driven by improved viral vectors for long-term transduction of neurons and the general safety of these vectors [46–50]. Many of these clinical programs are still in progress and will hopefully yield the identification of effective therapies in the future.

Yet despite this degree of progress, a great number of unmet neurological needs remain. Among these are the following disorders that either entirely lack any form of effective therapy or lack treatment approaches to slow or halt disease progression.

1. Alzheimer's disease, the most common neurodegenerative disorder.
2. Repair of damage caused by stroke, the second most common cause of death from all causes.
3. Amyotrophic lateral sclerosis, a disease that tragically robs individuals of movement and life in a very short time period.
4. Spinal cord injury, a disease that frequently strikes in the earlier years of life and imparts a lifetime of neurological disability.
5. Traumatic brain injury, a common consequence of combat and conflict that frequently affects young individuals and can lead to a lifetime of dysfunction.
6. Parkinson's disease, the second most common neurodegenerative disorder that often has a lengthy and highly disruptive disease course.
7. Inherited diseases of the nervous system, especially metabolic disorders that lead either to early death, mental retardation, or lifelong disability. These disorders include Rett Syndrome, Tay-Sachs disease, glycogen storage diseases, and several others.
8. Autism spectrum disorders, which are alarmingly common and encompass a range of clinical phenotypes and potential etiologies.

This book will present translational efforts in several of these arenas. The promise has never been greater, even as the nation's commitment to funding this research has waned in the last several years. Despite the lack of stronger commitment of funds, work is advancing at a rapid pace. Advances in treatment have been made in the last decade, and we expect the number of effective therapies that advance to regulatory approval to increase in the next decade.

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Part I
Molecular Approaches

Chapter 2
Gene Therapy of CNS Disorders Using Recombinant AAV Vectors

Giridhar Murlidharan, R. Jude Samulski, and Aravind Asokan

Abstract Corrective intervention for CNS disorders typically requires replenishment of depleted biomolecules (e.g., catabolic enzymes), protection of neurons and glia from premature death, or utilization of CNS cells as bio-factories for production of neurotransmitters or their biological precursors/cofactors. Gene therapy offers the ability to treat disorders in various organs by delivering therapeutic transgenes for regaining lost functionality. Adeno-associated viruses (AAV) have emerged as the vector of choice for CNS gene therapy. This chapter summarizes key observations made during preclinical and clinical evaluations of AAV vectors toward gene therapy of two broad categories of CNS disorders, namely metabolic storage disorders and movement disorders.

Keywords Adeno-associated virus (AAV) • Viral vectors • Gene therapy • Neurological disorders • Neurodegenerative diseases • Central nervous system

Introduction

The mammalian central nervous system (CNS) is a complex and precise connectivity of intertwining neurons nourished and supported by glial cells—astrocytes, oligodendrocytes, and microglia. Smooth functioning of the CNS is orchestrated by excitation and inhibition of neuronal firing/action potential, i.e., relay of potential difference traveling between the cell body (soma) and its projections at the axonal terminus/ni. Neurons are decorated with receptors for neurotransmitters

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like glutamate and gamma (γ)-amino butyric acid (GABA), which are specifically associated with excitatory or inhibitory responses [1].

While timely excitation and inhibition of regional subpopulations of neurons controls motor, behavioral, hormonal, sensory, and cognitive outcomes, unregulated neuronal activity and selective loss of neuronal or glial subgroups has been associated with CNS disorders. Such disorders can arise from drug abuse, injuries, genetic, epigenetic, and environmental factors. These debilitating events can manifest themselves as loss of motor skills, e.g., Parkinson's disease [2], epilepsy [3], Tourette's syndrome [4, 5], and amyotrophic lateral sclerosis (ALS) [6], and cognitive skills, e.g., Alzheimer's disease [6] and Autism [7], or can rarely even be lethal very early on in life, e.g., Canavan's disease [8]. A common theme among patients suffering from such diseases includes difficulties in performing day-to-day activities amounting to exceptional loss in quality of life, disruption of social life, heavy financial burden of treatment, and in most cases, absence of curative options.

To ameliorate such disease phenotypes, tremendous effort has been directed toward pharmacological regulation of events such as neurotransmitter signaling, e.g., by synthesizing receptor agonists/manipulating receptor domains by genetic reprogramming, etc. [9]. Although successful reversal of pathology is not common, such interventions often provide symptomatic relief for short periods of time and are therefore approved for clinical use. Unfortunately, side effects of pharmacological agents, irreversible nature of most genetic alterations, restricted ability of CNS cells to replenish themselves, and complicated clinical procedures make CNS disease therapy an exceptionally difficult endeavor. Viral vector-mediated gene therapy offers the ability to perform efficient in vivo gene transfer of therapeutic transgenes directed to the CNS. Specifically, replenishment of biomolecules that are depleted as a result of disease (e.g., catabolic enzymes), protection of neurons and glia from premature death, and even utilization of CNS cells as bio-factories for production of neurotransmitters and their biological cofactors have been demonstrated using different strategies of gene therapy.

Viral vectors, especially Adeno-associated viruses (AAV), have emerged as the vehicle of choice for supplying healthy cargo of therapeutic genes to the mammalian CNS [10–12]. The past few decades have witnessed consistent progress toward characterization and preclinical evaluations of AAV vectors in small/large animals and non-human primates (NHPs) [13]. Such efforts enable us to make informed decisions regarding parameters like viral serotype, route of administration, immune response, dosage, and biosafety and employ AAV vectors for therapeutic gene transfer in the clinic.

Recombinant AAV Vectors

Wild-type (wt) AAVs are non-enveloped parvoviruses ~25 nm in diameter with a single-stranded DNA (ssDNA) genome of ~4.7 kb [14]. WtAAV depends on genomic elements from larger helper viruses like adenovirus, herpes virus, papillomavirus, etc., for replication. The AAV genome is flanked by hairpin-like inverted terminal

repeats on 5′ and 3′ ends, and has two open reading frames encoding replication/rep, capsid/cap proteins, and an assembly activating protein (AAP) [15]. Due to alternative start codons, splicing variations, and differential promoter usage, the wtAAV genome encodes four rep and three cap protein variants [16]. The rep proteins are responsible for the smooth functioning of various events during viral life cycle, e.g., DNA replication, transcription, genome encapsidation, etc. The cap on the other hand encodes structural viral proteins 1–3 (VP1–3) in a ratio of 5:5:50 monomeric subunits building a 60mer $T=1$ icosahedral capsid. Understandably, the surface-exposed regions of the capsid impart characteristic features to different AAVs with regard to host cell surface attachment and antigenicity [15]. Such properties largely dictate the optimality of an AAV serotype for specific gene transfer applications [17]. More comprehensive analyses on AAV genomic elements and their functions can be found elsewhere [14, 16].

AAV gene therapy has come a long way since the initial discovery of the wild-type virus as a contaminant in adenoviral stocks. The combination of comprehensive understanding of AAV biology and some significant achievements in AAV vector engineering has equipped us to generate recombinant AAVs (rAAVs) [18–20]. This technology allows us to package the transgene of interest into an AAV serotype with desirable properties in either single-stranded (ss) or self-complementary (sc) configuration (Fig. 2.1). Such flexibility has enabled scientists to characterize an arsenal of natural and engineered AAVs as gene transfer vectors, for research and therapeutic applications. From the vantage point of CNS gene therapy, AAV vectors offer the following attractive features—(a) there is no conceivable evidence correlating AAV serotypes with pathogenicity/disease in animal models or human patients; (b) unlike other viruses, AAV is not highly immunogenic, with the

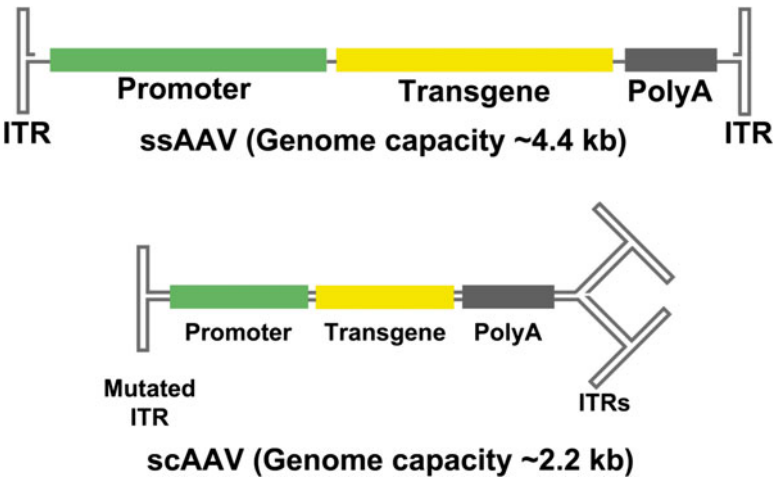

Fig. 2.1 Schematic representation of AAV genomes: the promoter, transgene, and polyadenylation signal (PolyA) are flanked by inverted terminal repeats (ITRs) in single-stranded (ss) or self-complementary (sc) configurations

immune response being usually restricted to generation of anti-capsid neutralizing antibodies [21]; (c) upon host nuclear entry, the genomic contents of AAV predominantly exist in an episomal form and utilize the cellular machinery for gene expression [14, 22, 23]. It is important to mention that a small percentage of AAV genomes have been reported to undergo highly specific insertion at the AAVS1 locus of human chromosome 19 [24]. In contrast, host genome integration is an integral part of the life cycles of other viral vectors like lentiviruses, adenoviruses, and herpes viruses [25]. Such events are often associated with insertional and frame shift mutageneses, sometimes resulting in carcinogenic outcomes for the host cells. (d) AAVs transduce both dividing and nondividing mammalian cells. This is especially important for CNS transduction where a majority of cells stop dividing once complete maturity is attained. (e) Lastly, direct brain administrations of different AAV serotypes result in distinctive patterns of cellular and regional gene expression in the CNS [26–34]. Such variations in AAV transduction profiles have been attributed to capsid–receptor interactions in different hosts [18]. For instance, AAV serotype 9 (AAV9) binds N-terminal galactose residues on the mammalian cell surfaces and shows extensive neuronal as well as glial transduction in animal models [35, 36]. On the other hand, AAV2, which utilizes Heparan sulfate as the primary receptor, demonstrates neuronal tropism and minimal spread from the injection site in the mammalian brain parenchyma [34, 37]. Another unique example is AAV4, which binds sialic acid and displays exclusive tropism for astrocytes at the injection site of the mammalian brain [38, 39].

In order to show differences in CNS transduction profiles of AAV vectors, we injected AAV4 or AAV9 packaging TdTomato (TdTom) fluorescent reporter gene driven by chicken β actin (CBA) promoter in the neonatal mouse brain. Intraventricular injections (white arrows, Fig. 2.2) of AAV4 resulted in TdTom expression (red) close to the site of injection (ependyma) (AAV4-CBA-TdTom, Fig. 2.2). On the other hand, AAV9 administration resulted in widespread TdTom expression across multiple regions brain parenchyma (AAV9-CBA-TdTom, Fig. 2.2). These results suggest that AAV vectors can be utilized to achieve both spatially restricted and widespread transgene expression in the CNS.

This chapter summarizes observations made during therapeutic applications of AAV vectors toward two broad categories of CNS disorders, namely Lysosomal storage disorders and Movement disorders.

Gene Therapy of CNS Disorders Arising from Metabolic Defects

Mammalian cells constantly break down complex biomaterials into simpler end products that make up for cellular nutrients or act as transient precursors for subsequent metabolic activities. Such biomaterials originate from dietary intake or preceding enzymatic degradations. Lysosomes are enzyme-rich digestive compartments inside a cell that are specifically designed to break down such buildup.

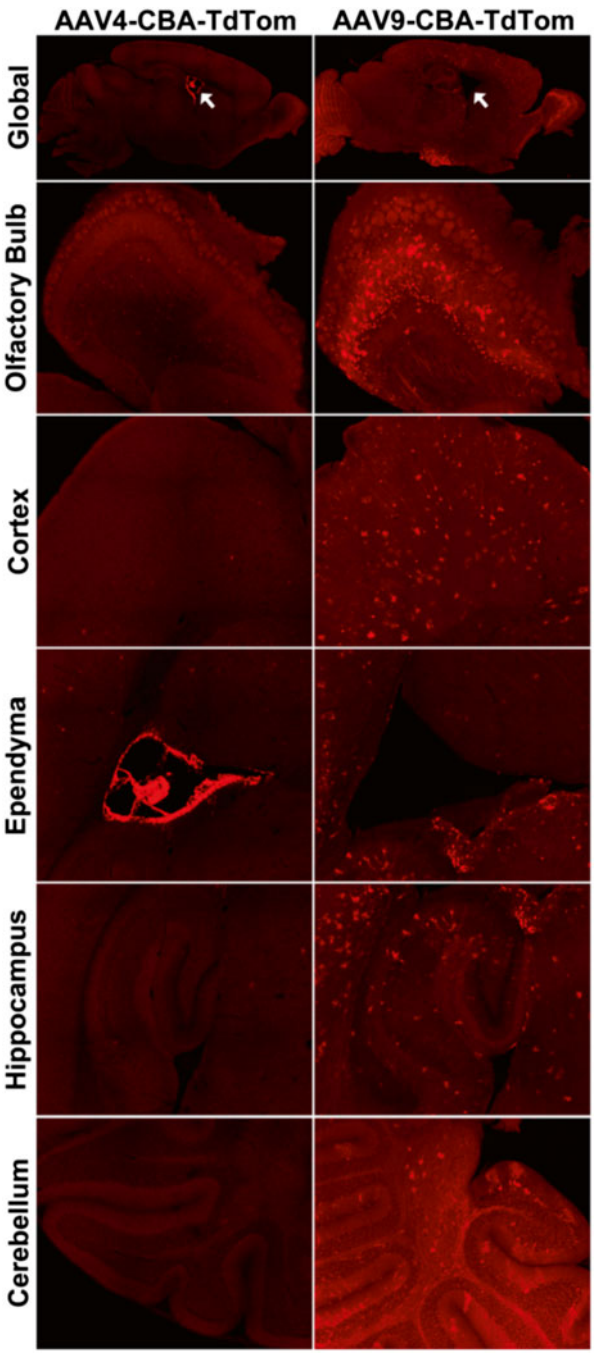

Fig. 2.2 Differential spread of CNS transduction achieved from AAV4 and AAV9 in neonatal mouse brain: postnatal day 0 (P0) mice were injected with 1×10^9 vg of AAV4 or AAV9 packaging a CBA-TdTom fluorescent reporter transgene into the left lateral ventricle. At 2 weeks post-injections, mice were sacrificed and paraformaldehyde fixed brains were sectioned. Brain sections were imaged using a Zeiss CLSM 700 confocal laser scanning microscope. Confocal micrographs show TdTom transgene expression (*red*) in 50 μm vibratome section of the mouse brain (global) and higher magnification images of individual regions in the rostrocaudal axis of the brain parenchyma

Understandably, dysfunctional lysosomes cause steady accrue of undigested enzymatic substrates, ultimately leading to cell death. Mammalian organs with continuously dividing cells are often able to compensate for this loss by replenishing themselves with new cells over time. On the other hand, once fully formed, the CNS undergoes very little cell division and reorganization. This makes it vulnerable to loss of functional tissue from such cellular distress. CNS disorders arising from metabolic storage burden have largely been classified as lysosomal storage disorders (LSDs). Another class of disorders called neuronal ceroid lipofuscinoses (NCLs) presents a similar phenotype and originates from the inability of the cells to break down the metabolite lipofuscins. The first association of a metabolic storage disorder with debilitating human disease was Pompe disease (Glycogen storage disease type II) characterized by severe progressive myopathy [40]. We are now cognizant of 50 LSDs that affect humans, most of which target CNS tissue [41]. It is worth mentioning that majority of LSDs and NCLs are transmitted in an autosomal recessive fashion, which accounts for their rare occurrence in the human population [42]. The molecular bases of many such disorders have been uncovered in the last few decades and have been reviewed elsewhere [41]. Such efforts have been important in the development of strategies to apply gene therapy toward disease treatment and amelioration. During normal conditions, catabolic enzymes utilize specific cellular macromolecules as substrates. Typically, LSDs and NCLs occur due to "loss of" or "mutations in" functional genes that encode such enzymes. Mucopolysacharidoses (MPS) are a broad range of LSDs branching from the common incapability of breaking down mucopolysaccharides resulting in fast deterioration of CNS milieu. Pharmacological enzyme replacement therapy (ERT) only provides short-lived and localized respite from the cellular buildup due to inefficient penetration of CNS tissue and inability of recombinant enzymes to cross checkpoints like the blood–brain barrier (BBB) [43]. A complete reversal of disease pathology demands continuous production and secretion of the lost enzymes in both CNS and peripheral organs. AAV gene therapy provides the necessary genomic elements to the patient's functional tissue for biosynthesis of lost enzyme(s) in a cell autonomous/nonautonomous fashion. In addition, the combinatorial spread of AAV vectors and the translated protein results in efficient penetration of the disease-affected CNS and peripheral organs. We have focused our discussion on major MPS disorders as case studies outlining progress toward understanding prominent metabolic storage disorders affecting the CNS and the use of AAV gene transfer toward their therapy.

MPS type VII (Sly disease) is a severely debilitating form of LSD that occurs as a result of accumulation of glycosaminoglycans (GAGs) due to the deficiency of β-glucuronidase (GUSB) enzyme. The disease symptoms include skeletal deformations, mental retardation, loss of sensory skills (vision and hearing), distorted features, and a short life span [44]. In one of the first attempts at utilizing AAV gene therapy toward amelioration of MPS VII in a mouse model, AAV2 vectors were engineered to package the GUSB transgene. Intravenous delivery of AAV2-GUSB led to reversal of

disease phenotypes pertaining to bone length, retinal function, and vacuole clearance in the MPS VII mouse model at an early age of postnatal day 2 (P2) [45, 46]. In theory, utilizing the bloodstream for delivering pharmacological agents to multiple organs is attractive, although it is not always the best route for administration of certain AAV serotypes for gene therapy.

Early studies conducted by Elliger and colleagues concluded that i.v. administration of the AAV2 vectors resulted in modest levels of CNS transduction and higher transgene expression in the peripheral tissues, i.e., heart and liver [47]. Recent research has demonstrated that AAV2 vectors are unable to cross the BBB [48]. Therefore, the clearance of GAG accumulation from CNS tissue observed in these initial studies using AAV2 is possibly due to secreted enzymes crossing the brain microvasculature or entering the parenchyma via aquaporin-mediated interstitial fluid clearance [18, 49]. Further supporting this argument, direct CNS administration of AAV2-GUSB via intrathecal injections in adult mice was demonstrated to achieve increased enzyme levels and decreased vacuole formations in the CNS tissue [47]. This study underscores the importance of pairing an AAV serotype with its optimal route of administration for CNS gene therapy applications. Widespread diffusion of the GUSB enzyme product in the CNS tissue was achieved by Skorupa and colleagues [50]. Their report demonstrated that direct injections of AAV2-GUSB into four sites in the adult rodent brain, namely striatum, cortex, thalamus, and hippocampus, achieve maximal spread of the enzyme. The authors reported clearance of lysosomal storage burden across the complete neuraxis in the ipsilateral hemisphere from such injections [50].

More recently, the use of multiple intracranial injections to achieve efficient biodistribution of therapeutic enzyme has been replaced by other strategies that are more amenable to clinical translation. To this end, a single striatal injection in the adult MPSVII-affected rat brain was shown to produce enzyme expression in 10 % of the brain volume leading to loss of the storage burden for ~16 weeks by Bosch and colleagues [51]. An alternative strategy toward achieving enhanced levels of transduction is to incorporate genetic elements like the promoter and enhancer, from other infectious mammalian viruses. The work of Sferra and colleagues demonstrated enhanced transduction efficiency of murine β-GUSB transgene, when driven by cytomegalovirus promoter–enhancer elements. ~50–240 % spread of enzyme expression across the CNS, resulting in metabolic storage benefit for up to 3 months of age, was reported by the authors [52].

Progressive loss of vision due to retinal degeneration is a characteristic clinical phenotype of MPSVII in humans. In an attempt to correct this pathology, AAV2-GUSB was injected into adult MPSVII mice via the intravitreal route by Hennig and colleagues [53]. The enzyme activity was observed in areas of the brain receiving visual inputs from the eye, e.g., thalamus and tectum. Interestingly, neighboring nonvisual areas like hippocampus and visual cortex also exhibited GUSB activity. The transduction profile suggested the combined role of synaptic vector transmission and diffusion of the translated product [53]. Understandably, expression of the GUSB

transgene in large three-dimensional spaces of the CNS is important for rapid lysosomal clearance and associated symptomatic benefits from MPSVII. Primate-derived AAV strains 7, 8, 9, and rh10 were injected into adult mouse brain regions cortex, striatum, thalamus, and hippocampus by Cearley and colleagues to assess their properties as CNS gene transfer vectors [26]. The authors reported that all tested serotypes showed preferential transduction of neurons and not astrocytes and oligodendrocytes. While AAV7 performed efficient gene transfer in cortex, thalamus, and hippocampus, AAV9 and AAV Rh.10 outperformed other serotypes in spread and transduction of both ipsilateral and contralateral hemispheres. Specifically, the assessment of AAV9-injected rodent brains demonstrated reversal of lysosomal clearance at 2 months post-injections in multiple regions of the brain. The authors also observed AAV9-mediated transduction across neuronal projections in the hippocampal commissure, providing supportive evidence for the vector's ability to undergo axonal transport to cover large distances in the CNS [26].

Although AAV-mediated gene therapy in the postnatal in vivo animal models successfully reverses clinical manifestations of LSDs, survival of such animals is still significantly lower than their wild-type counterparts. In one of the first attempts to introduce therapeutic intervention in an in vivo embryonic stage, AAV1 vector encoding the GUSB transgene was administered at embryonic day 15.5 (E15.5) by Karolewski and colleagues [54]. Vector-mediated CNS transduction resulting in enzymatic spread across the entire brain and spinal cord was observed. Low levels of GUSB activity were also observed in peripheral organs like liver, spleen, kidneys, and gonads. Interestingly, no vector genomes were detected at such off-target locations. The authors further discussed that the peripheral leakage of the enzyme was possibly due to uptake of CSF metabolites into the venous system. The CNS-specific therapeutic intervention was enough to confer benefit from lysosomal storage lesions for up to 1-year post-injections and improved the survival comparable to wild-type controls. Surprisingly, some of the clinical manifestations like facial and skeletal deformations were not rectified posttreatment. Such mixed results indicate room for improvement in areas like vector design and route of AAV administration [54].

Some of the most daunting challenges with clinical translation of gene therapy are related to surgical procedures during vector administration in the clinic. Major advances have been made toward reduction of invasiveness during therapeutic vector administration targeting the CNS. Intra-CSF injections like ICV injections, intracisternal injections (IC), and intrathecal lumbar puncture (IT) are all viable strategies to achieve maximal contact with the CNS tissue from a single dose of administration. In addition, viruses are also known to utilize axonal transport to cover long distances via inter-synaptic relay. AAV strains have different efficiencies and preferred direction(s) of movement across neuronal connections [55]. In this regard, utilizing regions of heavy afferent and efferent "wiring" within the brain can be a useful strategy. A comparative assessment of AAVs 1, 9, and Rh.10 injected into a major hub of neuronal projections, ventral tegmental area (VTA), was reported by Cearley and colleagues [56]. The authors compared VTA injections of the AAV

vectors to conventional striatal injections. While an increase in spread of transduction to distal regions of the brain was reported from all three AAV serotypes, the maximal spread was seen in case of AAV9 vectors. The authors then used the strategy to deliver GUSB transgene packaged in AAV9 vectors in the MPSVII mouse model. The study reported widespread transduction of the therapeutic transgene, leading to expanded biodistribution of the enzyme in the entire brain from a single 1 μl injection of ~10^{10} total viral genomes (vg) [56].

On the behavioral front, MPSVII patients display mental retardation due to neuronal and glial cell death. Frisella and colleagues hypothesized that AAV-mediated GUSB transgene delivery would achieve successful restoration of lost cognitive function. To this end, intracranial administration of AAV2-GUSB was performed in the MPSVII mice by the authors that led to long-lasting supply of the enzyme in the CNS. The authors then utilized the Morris water maze test to show that the mice treated with the AAV vector exhibit near wild-type levels of cognitive skills [57]. The loss of cognitive acumen is often related to dysfunction of the hippocampus, e.g., deteriorated learning and memory, difficulties in fear conditioning, etc. Preclinical evaluations of the extent of cognitive benefit seen in MPSVII mice due to AAV-mediated gene therapy were performed by Liu and colleagues [58]. The authors administered AAV5 packaging β-GUSB via bilateral intrastriatal injections in adult MPS VII mice. AAV5 has been previously demonstrated to be a highly neurotropic vector capable of transducing a larger area of the CNS than AAV2 [34]. AAV5-mediated intrastriatal delivery of the corrective GUSB transgene provided cognitive benefits in MPS VII mice as demonstrated in repeated acquisition and performance chamber (RAPC) assay. Specifically, the MPSVII-affected adult mice underwent RAPC assay before and after the vector administration. Post-administration, the mice displayed a significant reduction in the learning errors and latency period to reach the reward. Furthermore, the authors identified a specific loss of glutamate receptor on the surface of hippocampal neurons in the MPSVII-affected mice. Specifically, 40–60 % depletion of glutamate receptors GluR1, GluR2, and NR1 was observed in the hippocampal neurons which could be linked to the learning deficits. Furthermore, the authors demonstrated that the glutamate receptor levels of AAV5-βGUSB-treated mice were indeed restored comparable to the levels of heterozygous littermates, thereby providing the molecular basis of the therapeutic benefit [58].

MPSIIIB/Sanfilippo syndrome is a rare, genetically transmitted LSD where patients suffer from intracellular accumulation of glycosaminoglycan heparan sulfate. The disorder stems from the deficiency of α-N-acetylglucosaminidase (NaGlu) enzyme in the CNS. The disease features fast deterioration of CNS and peripheral tissues leading to severe mental retardation and premature death in patients [113], Metabolic and Molecular basis of inherited diseases). Enzyme biosynthesis using gene therapy confers successful protection of neuronal and peripheral tissue and provides symptomatic relief in the MPS IIIB mouse model. One of the earliest examples of therapeutic AAV gene transfer was reported by Fu and colleagues [59].

AAV2 vectors packaging the NaGlu transgene were engineered. The transgene was driven by either a constitutive cytomegalovirus (CMV) promoter or a neuron-specific enolase (NSE) promoter. Successful transduction of AAV2 in human MPS IIIB patient fibroblasts and mouse somatic and primary brain cells, resulting in significant degradation of GAGs, was demonstrated. A low dose (~10^7) of viral genomes of AAV2-NSE-NaGlu was then injected into the adult MPS IIIB mouse brain and successful NaGlu expression leading to correction of GAG storage in a broad CNS area was observed [59].

Cressant and colleagues assessed the effects of AAV-mediated gene transfer of the NaGlu transgene on the behavioral outcomes of MPS IIIB [60]. It is now clear that certain AAV serotypes like AAV2 have the preferential ability to undergo axonal transport in the anterograde direction [61]. Regions of extensive synaptic connectivity are attractive sites of injection for AAVs packaging corrective transgenes to target a large area of the brain from a single injection. In the mammalian CNS, axonal projections to multiple regions originate at the caudate putamen. In this report, the authors compared putaminal injections of AAV2 and AAV5 vectors in adult 6-week-old MPS IIIB mice and demonstrated that AAV5-mediated transduction spreads more than AAV2 [60]. AAV5 binds *N*-linked sialic acid as cell surface attachment factors, whereas AAV2 requires the availability of heparan sulfate proteoglycans (HSPG) to perform successful gene transfer [37, 38]. The interaction of AAV2 with its cognate receptor (HSPG) is one of the rare occurrences where cellular receptor binding is detrimental to the spread of the AAV vectors in the CNS [18]. This phenomenon might explain the enhanced ability of AAV5 vectors to spread and transduce CNS tissue in the distant regions from the site of injection. Both AAV vector injections resulted in the enzyme bioavailability above untreated controls leading to reversal of disease phenotype in the neurons, microglia, and perivascular cells. Additionally, a complete reversal of behavioral symptoms from both AAV2 and AAV5 treatments was reported [60]. The assessment of behavioral recovery was performed using a circadian cycle controlled open field test. Parameters such as mouse activity during light and dark time periods and explorative and habituated navigations were monitored during the course of the sessions. To summarize, these tests provide a fair assessment of the success of gene therapy toward reversal of cellular pathology and behavioral outcomes seen in MPS IIIB patients featuring anxiety, restlessness, hyper-excitability, and aggressiveness [60].

Many natural and engineered AAV isolates are being discovered with attractive properties like the ability to spread and transduce large CNS volumes. In an attempt to achieve widespread correction of the MPS IIIB disease pathology in the CNS, AAV2-mediated NaGlu gene transfer was performed by Fu and colleagues in BBB compromised mice in vivo [62]. Specifically, the authors utilized intra-arterial injection of mannitol post-AAV administration that led to transient opening of the BBB. While there is no measurable permanent damage inflicted upon the BBB due to this treatment, efficient entry of viruses, antibodies, and large macromolecules has been widely documented due to such treatment [62–64]. The authors

demonstrated that combined intravascular and intra-cerebrospinal fluid (CSF) vector administration led to successful and long-term correction of the disease pathology and a significant increase in the life span of the mice (from 7.9–11.3 months in untreated mice to 11.1–19.5 months in treated mice). Simultaneous use of CSF and blood connectivity led to successful gene transfer not only in the CNS tissue but in peripheral organs as well. Virtually the entire brain and spinal cord tissue are connected with the combination of the CSF and blood vessels. The CSF constantly provides nutrients and molecular signals to, and drains interstitial fluids from, the brain and spinal cord tissue via the sub-arachnoid space, cerebral ventricles, cerebellar foramen, and the cisterna magna [49, 65–67]. The blood vessels on the other hand provide constant supply of oxygenated blood due to the combination of arterial influx and venous efflux in the CNS tissue. The authors of this study speculated that the success of their therapeutic AAV administration could be attributed to the routes of administration [62]. The transient opening of the BBB clearly assisted the CNS spread of AAV vectors. Moving forward, the time span between intra-arterial mannitol infusion and the i.v. administration of AAV vectors was optimized by McCarty and colleagues. The authors demonstrated that injections performed exactly 8 min after the mannitol infusions led to significantly enhanced viral transduction resulting in reversal of the disease phenotype in the MPS IIIB mice [64].

Combinatorial use of other strategies of therapeutic intervention can have a synergistic effect on AAV-mediated gene therapy of the CNS. Exploring such a hypothesis, AAV gene transfer in combination with bone marrow transplant and assessment of correction of MPS IIIB was performed by Heldermon and colleagues in mice. Specifically, the authors performed intracranial AAV5-NaGlu administration with/without the transplant of NaGlu-transduced bone marrow cells in the MPS IIIB mouse model. The bone marrow transplant (BMT), by itself, was the least efficacious of all three strategies and the report surprisingly concluded an antagonistic effect of the combination treatment on survival and motor skills in the disease-affected mice [68]. Such outcomes provide cautious optimism and direction for the future use of AAV toward CNS gene therapy of metabolic storage disorders.

Gene Therapy of Movement Disorders

The timely firing of neurons projecting within corticostriatal, nigrostriatal, and thalamocortical circuits of the brain orchestrates events leading to motor control. Under the umbrella of movement disorders, therapeutic gene transfer using rAAVs has shown some promise in animal models of Huntington's disease (HD) and Parkinson's disease (PD). For the purpose of this chapter, we will focus on PD and assess the use of AAV vectors in understanding and treating the multifactorial CNS disorder. Familial, environmental, and idiopathic factors result in selective loss of dopaminergic (DA) neurons leading to Parkinsonism [69]. PD patients exhibit bradykinesia

(slow movements), akinesia (no movements), and tremors among other symptoms exemplifying disrupted motor function. During normal conditions, DA neurons at the basal ganglia (Substantia Nigra Pars Compacta (SNPc) region) send inhibitory inputs to the subthalamic nucleus (STN) through enhanced GABA signaling [2]. Such inhibitory inputs received at the STN are important for the controlled excitation of the projections to motor-associated regions of the cortex. Detailed descriptions of the neuronal subtypes and comprehensive analyses of the circuitry that governs the states of normalcy and disease have been reviewed elsewhere [2, 69]. On a cellular level, PD features loss of DA neurons at the SNPc leading to reduced inhibition of the STN causing unregulated inputs reaching motor areas further downstream. Understandably, the dysfunction of two major checkpoints, i.e., loss of DA neurons/dopamine and reduction of GABAergic input to the STN, results in disease. Over the years, gene therapy research has focused on both of these checkpoints to develop strategies that can reverse PD pathology.

One of the hallmarks of PD is the appearance of aggregated synaptic protein alpha-synuclein (α-syn) in the surviving dopaminergic neurons [70, 71]. Such aggregations called "Lewy bodies" are not restricted to PD. A similar phenotype occurs in Alzheimer's disease (AD) where aggregations of the proteins amyloid-β (Aβ) and tau have been associated with loss of hippocampal neurons resulting in learning and memory defects [72]. In case of PD, the phosphorylation of the protein α-syn at the Serine-129 position has been associated with potent disease pathology [73–76]. A key mechanistic insight of the association between PD pathology and α-syn aggregation was provided by the work of Gorbatyuk and colleagues [77]. The authors performed nigral injections of rats with AAV5 vectors packaging three versions of α-syn, i.e., wt α-syn, phosphorylated α-syn S129A, or non-phosphorylated α-syn S129D. The enzyme TH is a biomarker for DA neurons as it is required for the conversion of L-tyrosine to levodopa, a precursor of dopamine [78]. The study demonstrated that the AAV-mediated delivery of S129A mutant was highly toxic to the TH immunopositive (TH+) DA neurons resulting in loss of striatal dopamine levels in the brain. Intermediate loss of TH+ neurons was also observed due to AAV-mediated overexpression of wild-type α-syn. More importantly, the study reported that the unphosphorylated form of α-syn (S129D) was not toxic to dopaminergic neurons and was incapable of causing PD pathology. In summary, this in vivo study utilized AAV vectors to demonstrate that PD pathology due to α-syn aggregation is dependent on its phosphorylation state at the S129 position [77].

The use of AAV vectors to achieve controlled biosynthesis of dopamine in vivo has been reported by Li and colleagues [79]. Briefly, AAV2 vectors packaging dopamine synthesizing enzyme TH, flanked by LoxP loci, were generated. In the event of Cre recombination, the transgene expression would be lost, leading to a loss of TH gene expression in the transduced neurons. To incorporate temporal control of TH expression in their system, the authors packaged 4-hydroxytamoxifen inducible version of Cre ($CreER^{TS}$) into another AAV vector [80]. The authors speculated that such AAV-mediated regulation of TH expression would alter the

dopamine levels in both in vitro and in vivo settings. Transfection of AAV2 vectors packaging the LoxP-flanked TH transgene led to efficient production of dopamine which was significantly lost after subsequent superinfection of AAV2-Cre/CreER^{TS} recombinases in cell culture. This confirmed proper functioning of constitutively active and inducible Cre systems in vitro. The authors then tested their system in Parkinsonian rats. Specifically, neurotoxin 6-hydroxydopamine (6-OHDA) was injected into rat brain to induce lesions in the dopaminergic areas of the CNS, generating a Parkinsonian rat model. Such rats then received individual/combinations of the abovementioned AAV vectors in the striatum and were assessed for biochemical and behavioral outcomes associated with PD. Significantly increased dopamine production in the animals that received AAV2 vectors packaging TH encoding transgene was reported. Subsequent Cre-recombinase-mediated loss of dopamine production led to significant disruption of motor skills during apomorphine-induced rotation tests and spontaneous limb movement tests.

During low levels of dopamine availability, the compensatory mechanism employed by the brain involves conversion of dopamine precursor levodopa into usable dopamine using L-amino acid decarboxylase (AADC) enzyme. The Cre-recombinase-mediated loss of TH expression led to significant reduction of dopamine levels, but did not affect the cellular expression of AADC in the rats that received oral levodopa. This suggested that the inherent compensatory mechanism of dopamine production is unaffected by the AAV treatments. These results demonstrated a system wherein AAV gene therapy was used to achieve temporally controlled induction and rescue of PD pathology in vivo [79].

Gene therapy-mediated replenishment of the levels of AADC enzyme in the brain is a potential strategy to ameliorate PD pathology. A phase 1 clinical trial reported the use of tracer-dependent positron emission tomography (PET) to track the spread of AADC transgene delivered using AAV2 in six PD patients who underwent putaminal injections of the vectors [81]. The patients were given oral levodopa for continuous supply of the enzymatic substrate. PET analysis revealed a 56 % increase in the bioavailability of AADC for up to 96 weeks post-injections. Six months post-injections, the authors observed ~46 % improved Unified Parkinson's Disease Rating Scale scores. These results show that the AAV vectors can be utilized for potential therapeutic intervention for PD pathology, but is also safe and well tolerated in a human disease setting [81].

The combinatorial intracranial delivery of the therapeutic enzyme AADC along with oral administration of levodopa is not free of side effects. Continuous intake of levodopa leads to complications associated with loss of impulse control leading to uncontrolled motions (Dyskinesia) among other symptoms [82–84]. Cederfjall and colleagues hypothesized that gene therapy can be used to achieve focused biosynthesis of dopamine in therapeutically relevant regions of the brain [85]. For this, two rate-limiting enzymes involved in the conversion of tyrosine from human diet to DOPA (3,4-Dihydroxyphenylalanine) were packaged in the same AAV vector. Intrastriatal delivery of AAV5 vectors encoding the transgenes tyrosine

hydroxylase (TH) and GTP-cyclohydrolase-1 (GCH1) led to efficient production of DOPA and its cofactor 5,6,7,8-tetrahydro-L-biopterin (BH4), respectively. The previously discussed 6-OHDA-induced PD rat model was used to assess the efficacy of AAV-mediated gene therapy. Efficient expression of TH and GCH1 was reported at the striatum and SN. The authors speculated anterograde transport of the vector to be responsible for this transgene expression profile in the rat brains. AAV-treated PD rats in the study exhibited supraphysiological levels of BH4 expression along with accumulation of synthesized DOPA in the forebrain regions, possibly due to the saturation of available AADC enzyme. Furthermore, AAV-treated rats exhibited motor and behavioral benefits from PD pathology, not seen in the lesion control animals. Specifically, the motor skills were compared using amphetamine-apomorphine-induced rotation tests and corridor and staircase tests. Significant therapeutic benefit with forelimb akinesia, sensorimotor control, and symmetry of movements was reported. In short, widespread biochemical reversal of PD pathology (DOPA production) in the AAV5-treated 6-OHDA lesioned rat brains resulting in functional recovery of motor control was observed [85]. Another comprehensive study was recently conducted by the same research group utilizing administration of the aforementioned AAV5 vectors. This study surprisingly reported the inability of their previous strategy of vector administration used in the PD rat model to directly translate therapeutic benefit in higher order mammals. In NHPs, such AAV5 administration led to increase in the GCH1 levels but not TH levels. It was speculated that other important parameters like promoter/enhancer elements were to be optimized so as to dissect the molecular basis of the incoherence seen between gene therapy of rats and monkeys [86].

In addition to accentuation of dopamine levels by delivering enzymes that partake in its biosynthesis, gene therapy could be used to reverse another aspect of PD pathology, neurodegeneration. Gasmi and colleagues have extensively characterized the neuroprotective factor Neurturin (NTN) or Glia-derived neurotropic factor (GDNF) toward amelioration of PD pathology [87]. The authors packaged NTN in AAV2 vectors and performed intra-striatal injections in a 6-OHDA-induced rat model of PD. NTN gene expression was reported as early as 2 days post-injections and lasted till the last time point of the study, i.e., 1-year post-administration. Further evaluation of the kinetics of the AAV gene transfer reported that both the bioavailability of the enzyme and the expression of the transgene stabilized in 4 weeks poststriatal injections. The effect of NTN transduction on the dopaminergic neurons was further evaluated at the SN via immunostaining for TH. A significant increase ($p < 0.001$) in the neuroprotection of the TH-immunopositive (TH+) SN neurons as compared to the control animals was reported. Similar promising results were reported during intra-striatal injections of AAV2 packaging the neurotrophic factor Pleiotrophin (PTN). PTN has a protective and nourishing effect toward nigrostriatal dopaminergic neurons lost during PD. Studies have shown that PTN expression is associated with differentiation of mesencephalic TH+ neurons and neuroprotection of surviving DA neurons during PD [88, 89]. To utilize such properties of PTN toward gene therapy of PD, intrastriatal delivery of AAV1 encoding the PTN transgenes

was performed by Gombash and colleagues [90]. Interestingly, this led to restricted transduction of neurons at the striatum and SNPc leading to efficient neuroprotection of DA neurons and reversal of PD phenotype in the 6-OHDA rat model.

It is clear that the injection of neuroprotective agents like GDNF has a stimulatory effect on DA neuronal growth at the site of injection. While functional reversal of PD pathology from intrastriatal injections of AAV vectors packaging GDNF has been shown, SN injections of such AAV vectors have been associated with "aberrant sprouting." Specifically, intracranial administration of viral vectors packaging GDNF has been shown to cause innervation of DA neurons in unspecific regions around the site of injection [91]. The unprecedented increase of DA synthesizing neurons at random projection regions has been associated with counter-beneficial behavioral side effects in the animals [92].

Fetal ventral mesencephalic (VM) cells are precursors of DA neurons in the mammalian CNS. Direct injections of fetal VM tissue implants have shown reversal of PD pathology via integration and striatal innervation of mature DA neurons. Functional turnover of dopamine biosynthesis leading to reversal of behavioral pathology in PD animal models has been reported from such treatments [93–95]. A combination of gene and cell therapy strategies was used by Redmond Jr. and colleagues in an attempt to augment the benefits individually achieved by both [96]. AAV5 vectors packaging the GDNF transgene were co-administered with VM tissue grafts into the caudate and putamen striatal regions of MPTP-induced PD model of NHPs. A head-to-head comparison of the gene and cell therapy based systems individually and in tandem was performed. Striatal levels of both DA and GDNF were significantly higher in animals that received the dual treatment in comparison to either individual procedure. Interestingly, during the phenotypic evaluation conducted over a span of 8 months posttreatments, the dual treatment did not show a significant increase in the amelioration of PD pathology as compared to the singular treatments. The authors speculated that these discrepancies in the biochemical and functional outcomes could be accounted for by events like downregulated TH expression at innervations of DA termini and aberrant sprouting events in the treated NHPs [96]. These results indicate that gene and cell therapeutic interventions that are autonomously capable of reversing PD pathology do not always complement one another. Such results need to be taken into consideration before clinical translation of such combinatorial procedures.

Human erythropoietin (EPO) enhances the production of red blood cells and is therefore an unlikely candidate for gene therapy of neurodegenerative disorders in CNS. However, recent research has indicated strong neuroprotection of dopaminergic neurons achieved by EPO expression in the brain. Mechanistically, the protein is known to have anti-inflammatory and anti-apoptotic effects among others leading to protection from neuronal loss during experimental neurodegeneration (from hypoxia-induced ischemia) or toxic insults (e.g., MPTP and 6-OHDA abuse) in vivo [97–100]. In an attempt to utilize the aforementioned therapeutic properties of the EPO protein, Xue and colleagues packaged human EPO transgene into AAV9 vectors and injected them into the rat striatum [101]. The authors reported

neuroprotection of DA neurons due to widespread transduction of EPO in the striatum and SN. The behavioral PD pathology was also attenuated in the rats injected with AAV9-EPO, as demonstrated in the rotation test and the test for spontaneous use of forelimbs.

Taken together, such experimental and clinical outcomes have characterized three main strategies routinely used in gene therapy of PD, i.e., dopamine biosynthesis [102]; functional growth, protection, and innervation of DA neurons [103, 104]; and neurochemical inhibition of STN [105, 106]. Clearly, it is important to restrict the aforementioned processes to functionally relevant regions of the brain by choosing the optimal site of injection, dosage, and the right AAV serotype during PD gene therapy. Other challenges associated with CNS gene therapy in the clinic include neutralizing antibodies, long-term bioactivity, and aggregation of the protein product. It is important to note that none of these concerns have posed serious adverse effects in the last decade of research in PD gene therapy [107–111]. A comprehensive perspective on gene therapy of PD with special focus on predictive animal models, clinical trial design, safety, patient selection, and the current limitations has been recently provided by Bartus and colleagues [112].

Summary

The success of CNS gene therapy hinges on a thorough understanding of successes achieved and challenges faced during experimental and clinical administrations of gene transfer vectors. In this regard, although AAV vector-mediated gene transfer in the CNS has demonstrated safe and successful delivery of proteins, the efficacy of gene therapy reported in the last decade of clinical trials has not met the expectations of researchers and clinicians. There remains a critical need for predictive in vivo models of neurodegenerative disorders, establishing correlation between preclinical studies conducted in rodent and primate animal models, and recruitment of representative patient cohorts for clinical trials and placebo effects. Moving forward, it is particularly important to invest time and effort toward various aspects of AAV vector design, development of biomarkers, and animal models to overcome the existing roadblocks. Some of the prominent applications of AAV-mediated CNS gene therapy that are currently undergoing various stages of clinical trials as reported in the National Institutes of Health's database (https://clinicaltrials.gov/) have been listed in Table 2.1. Although we were unable to discuss gene therapy of many other CNS disorders and several other outstanding scientific contributions, through this chapter, we have attempted to bring clarity to the advantages and challenges associated with the therapeutic use of AAV vectors in the CNS.

Table 2.1 Clinical gene therapy of some neurological disorders using AAV vectors

Category	Disorder	Transgene	AAV serotype, route and dosage	Clinical status	Summary
Metabolic storage disorders	Batten's disease	Human CLN2 gene	AAV2—Intracranial injections	Phase 1 (NCT00151216)	Expression of CLN2 transgene to replenish the levels of tripeptidyl transferase-1 (TPP1) enzyme
			AAV Rh.10—Intracranial injections—2.85 or 9×10^{11} vg	Phase 2 (NCT01414985)	
	Pompe disease	Human acid alpha-glucosidase (GAA) gene	AAV9—Intramuscular injections—5×10^{9} vg	Phase 1 (NCT02240407)	The enzyme GAA degrades cellular accumulation of glycogen which leads to severe neuromuscular pathology
			AAV1—Intramuscular injections—1-5×10^{12} vg	Phase 2 (NCT00976352)	
	MPS III A (Sanfilippo's type A syndrome)	Human SGSH and SUMF1 genes	AAV Rh.10—Intracranial injections	Phase 2 (NCT01474343)	Expression of N-Sulfoglucosamine Sulfohydrolase and Sulfatase modifying factor 1 leads to degradation of glycosaminoglycans (GAGs) accumulations in the CNS tissue
	Acute intermittent porphyria	Porphobilinogen deaminase (PBDG) gene	AAV5—Intravenous injections	Phase 1 (NCT02082860)	PBDG enzyme deficiency blocks heme biosynthesis and causes severe neuropathic symptoms ranging from abdominal pain to seizures and psychotic episodes

(continued)

Table 2.1 (continued)

Category	Disorder	Transgene	AAV serotype, route and dosage	Clinical status	Summary
Movement disorders	Parkinson's disease	Human aromatic L-amino acid decarboxylase (AADC)	AAV2—Intracranial I (striatal injections)—9×10^{10}–3×10^{11} vg total	Phase 1 (NCT00229736)	AADC-mediated conversion of orally administered levodopa into dopamine
			AAV2—Intracranial (striatal injections)—7.5×10^{11}–2.3×10^{12} vg total	Phase 2 (NCT01973543)	
		Glial cell line-derived neurotrophic factor (GDNF)	AAV2—Intracranial (striatal injections) —9×10^{10}–3×10^{12} vg total	Phase 1 (NCT01621581)	Neuroprotection of dopaminergic neurons with GDNF
		Neurotrophic factor neurturin (NTN)	AAV2—Intracranial (putaminal injections)	Phase 1 (NCT00252850)	Neurturin-mediated neuroprotection of dopaminergic neurons
			AAV2—Intracranial (putaminal and substantia nigral injections)—4-5×10^{11} vg or $24\times10^{11}\times$vg	Phase 1 (NCT00985517)	
		Glutamic acid decarboxylase (GAD)	AAV-GAD intracranial injections (subthalamic nucleus)	Phase 1 (NCT00195143)	GAD expression in the STN has an inhibitory effect due to GABA production

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Chapter 3
NGF and BDNF Gene Therapy for Alzheimer's Disease

Mark H. Tuszynski and Alan H. Nagahara

Abstract Nervous system growth factors have extensive effects on neuronal function and survival. Nerve Growth Factor (NGF) prevents the death and stimulates the function of basal forebrain cholinergic neurons in correlational models of Alzheimer's disease (AD), leading to its translation to Phase 1 and 2 human clinical trials. Separately, Brain-Derived Neurotrophic Factor (BDNF) influences the survival and function of entorhinal cortical and hippocampal neurons in several animal models of AD, including transgenic mutant APP-expressing mice, aged and lesioned rats, and aged and lesioned primates. These beneficial effects occur independent of detectable alterations in beta amyloid load. We are currently examining the extended safety and tolerability of BDNF gene delivery to the entorhinal cortex in animal studies, leading to specific targeting of short-term memory loss in upcoming human AD trials. Collectively, a large body of research suggests that growth factor therapy represents an alternative to amyloid-modifying drugs for preventing neuronal degeneration and stimulating neuronal function in Alzheimer's disease, with the potential to reduce disease progression.

Keywords Alzheimer's disease • Nerve growth factor (NGF) • Brain-derived growth factor (BDNF) • Neurodegeneration • Cholinergic basal forebrain • Entorhinal cortex • Clinical trials

Introduction

Gene therapy is a developing technology that has the potential to treat a number of human diseases, including neurodegenerative disorders. Gene therapy allows the expression of specific proteins in defined types of cells with anatomical specificity in the central nervous system; this approach might be useful for the treatment of

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neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, spinal cord injury, and others. The most prevalent neurodegenerative disorder is Alzheimer's disease (AD), currently afflicting about 36 million people worldwide. Existing treatments for AD are only marginally effective and fail to influence disease progression. Treatment approaches to AD that target modulation of beta amyloid production are undergoing advanced clinical testing, but these drugs have to date not altered the course of established disease, leading to current efforts to treat AD *before* symptom onset. Thus, alternative approaches to therapy are needed: growth factor gene therapy exhibits efficacy in AD animal models when treatment is begun after disease onset. Moreover, by targeting a mechanism other than amyloid, growth factor gene therapy offers the potential of combination therapy with amyloid-modifying drugs. This chapter reviews the use of gene therapy with neurotrophic factors in the treatment of this complex disorder.

Alzheimer's Disease

Background

Alois Alzheimer, a German psychiatrist and neuropathologist, first described AD in 1906. It is now the most prevalent neurodegenerative disease. Five million people in the United States are currently affected by AD, and an estimated 36 million people are affected worldwide. The age of AD onset is past 65 years of age in 95 % of cases [1]. With the expansion of the aged population, the number of AD victims is predicted to be 13.8 million people in the United States and 115 million people worldwide by 2050 [1, 2]. Annual estimated costs of AD care in the United States alone are estimated over $200 billion, not including the cost of care provided by family members [1]. Unfortunately, there are no substantially effective therapies either to improve AD symptoms or to slow progression of the disease. While cholinesterase inhibitors and the drug memantine (Namenda; a putative glutamate antagonist) yield detectable symptomatic benefits, the overall impact of these drugs is small [3–5]. Better therapies are clearly needed in general, and the development of disease-modifying drugs in particular remains an important goal of the field.

Symptoms and Neuropathology

Short-term memory loss is the cardinal symptom of AD, and may occur in isolation in the earliest symptomatic stages of the disease. As the disease progresses, other cognitive domains become impaired including executive function, visuospatial skills, language, perceptual processing, and attention [6]. Behavioral dysfunction can also occur, including aggression, agitation, psychosis, and motor dysfunction

[7]. Diagnosis of AD currently rests primarily on cognitive testing. However, a major focus of research is the development of biomarkers of AD at its earliest stages, including presymptomatic states. Recent evidence indicates that changes in the brain can be visualized by PET imaging of amyloid plaques [8] and possibly tau [9], hippocampal atrophy on quantitative MRI [10], and changes in cerebrospinal fluid and blood biomarkers [1, 11–13]. However, the current diagnosis of AD in clinical practice still rests on predominantly clinical measures, and is typically made at a stage in which patients already exhibit short-term memory loss; at this stage of the disease, there is already substantial damage to the entorhinal–hippocampal region [14–17].

Neuropathological features of AD in the brain consist of hallmark features of amyloid plaque formation and intraneuronal neurofibrillary degeneration (Fig. 3.1). With this, there are a loss of synapses and then neurons. These neuropathological features are found in many regions of the brain at later stages of the disease. However, the initial neuropathology starts in the entorhinal cortex and hippocampus, spreading to other brain regions [18–20]. The abnormal processing and subsequent accumulation of beta amyloid peptide constitutes one essential component of pathological degeneration [21]. Approximately 5 % of AD cases represent an early-onset familial form of the disease related to mutations of either the amyloid precursor protein gene itself or presenilin genes that result in accumulation of beta amyloid in the brain [22]. Neurofibrillary tangles consist of accumulations of hyperphosphorylated Tau protein in neurons [23]. Accumulation of Tau is an essential feature of a diagnosis of AD, and Tau accumulation is also found in other neurodegenerative disorders including frontotemporal dementia and multisystem atrophies [24].

The loss of synaptic connections represents a major neuropathological feature of AD that occurs early in the disease process and is most directly associated with cognitive decline [25]. Synapse loss also correlates eventually with the extent of beta amyloid accumulation [26, 27]. Alzheimer's disease is further associated with

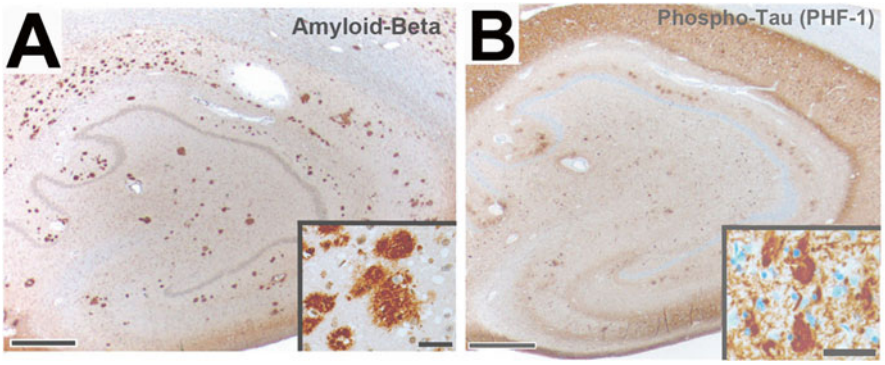

Fig. 3.1 Alzheimer's disease pathology. (**a**) Amyloid deposition in the hippocampus of a typical AD patient. Inset illustrates the presence of dense-core and diffuse plaques. (**b**) Neurofibrillary tangles in the hippocampus of an AD patient. Inset shows neurofibrillary tangles and dense tau pathology

a loss of neurotrophic factor levels or availability in the brain [28–31], which in turn directly influences synaptic function [32, 33]. The loss of synaptic connections may be critical, since the loss is highly correlated to functional decline in AD [27, 34]. A number of secondary pathological changes also occur in AD including inflammation, mitochondrial dysfunction, increased oxidative stress, dysregulation of cellular calcium homeostasis, and axonal transport defects [29, 35–37].

The progressive nature of cognitive decline in AD reflects the ongoing neuropathology in localized brain regions [18, 19]. The entorhinal cortex is the first brain region to undergo neurodegeneration in initial stages of AD [18, 19]. Early memory deficits in AD are thought to reflect this entorhinal and hippocampal pathology, as indicated by loss of cells [14, 16] and synaptics between the entorhinal cortex and hippocampus [34, 38]. Volumetric studies using MRI confirm early changes in the entorhinal cortex and hippocampus in the mild cognitive impairment stage of AD [39–42]. As the disease advances, neuropathology progresses to other cortical areas and limbic structures. The progression of AD to other brain regions represents patterns of anatomical connectivity [20, 43, 44]. Recent evidence indicates that patterns of anatomical spread might be related to trans-neuronal spread of tau proteins [45, 46]. Thus, slowing the progression of AD in the brain regions affected during the early stages might represent an effective treatment strategy. This is an objective of the BDNF gene therapy program in AD, as described below.

Numerous other brain regions are also damaged in AD. The basal forebrain cholinergic system is among these and may have particular importance [47, 48]. Cholinergic neurons of the basal forebrain regulate neural activity in the hippocampus, neocortex, and other cortical regions and exert an important role in attention and memory function [49, 50]. Indeed, one of only two classes of drugs approved for the treatment of AD compensates for cholinergic degeneration by inhibiting cholinesterase activity and elevating acetylcholine levels [3, 51]. However, the efficacy of this class of drugs is relatively mild, in part due to dose-limiting side affects resulting from nonspecific activation of cholinergic activity systemically, rather than a focused effect on active cortical systems. Hypothetically, a therapy that *preserves* cholinergic neurons could represent a superior therapy in AD: this is the objective of the nerve growth factor (NGF) gene therapy program in AD, described below.

The only other class of drug approved for the treatment of AD in the United States is the partial NMDA antagonist memantine (Namenda) [5]. Memantine, like cholinesterase inhibitors, targets diverse neurons throughout the brain. However, the overall impact of this drug on clinical symptoms is also relatively mild, and memantine does not change disease progression [51].

Several clinical trials are now in progress that aim to reduce beta amyloid accumulation in the brain and accordingly reduce AD progression. Data in amyloidogenic mouse models heralded this line of work [52]. Amyloid precursor protein is a transmembrane protein that can be cleaved by beta- and gamma-secretases,

resulting in accummulation of "mature" beta amyloid fragments of 40–42 amino acids together with the activation of intracellular caspace activity. To reduce cleavage of amyloid precursor protein and reduce beta amyloid accumulation, secretases can be inhibited or modulated [51] (see Chap. 19). Alpha secretases also cleave amyloid precursor protein to produce a non-amyloidogenic peptide, and drugs aimed at increasing alpha secretase activity represent another therapeutic strategy. Clinical trials with these drugs in AD patients to date have been disappointing, but work is ongoing with modified versions of the drugs and in patients with earlier stage or even presymptomatic AD.

Another way to target beta amyloid pathology in AD is to clear it from the brain. So-called immunotherapy can either generate an immune response to beta amyloid or infuse antibodies directed against beta amyloid to enhance its removal from the brain [51]. The first such clinical trial in AD was performed in 2002 and actively immunized patients with the 42 amino acid Aβ1–42 peptide, together with an immune adjuvant to stimulate antibody production [53–55]. 6 % of vaccinated AD patients developed an apparent autoimmune meningoencephalitis, resulting in a halt to the trial [53, 56]. However, patients who generated an anti-Aβ antibody response showed reduced levels of tau in the CSF [53, 57]. The brains of a small cohort of patients from the trial demonstrated a significant reduction in amyloid plaques in the brain compared to non-treated controls, but disappointingly showed no evident cognitive benefit [54]. Current clinical trials in AD are excluding immune adjuvants when administering immunizing peptides, are using different immunizing peptides, or are directly infusing monoclonal antibodies directed against Aβ (passive immunization). Two clinical trials of Aβ monoclonal antibody therapy (bapineuzumab and solanezumab) failed to achieve statistically significant clinical benefits on preplanned primary outcome measures [56], but raised the possibility of potential benefit in patients with more mild disease. Given the apparently pivotal role of beta amyloid in the pathogenesis of AD, and the potential signal of benefit of anti-amyloid therapy in patients with milder disease, the hypothesis has been raised that treatment may have to begin at very early stages of the disease, perhaps even in patients at risk for AD who have not yet developed clinical symptoms. Indeed, since subsequent clinical trials of anti-amyloid therapies have generally shown good safety, several large clinical trials in presymptomatic patients with familial AD or high amyloid plaque load have begun [54]. These are very important studies.

Additional drugs are undergoing testing in AD clinical trials, targeting other components of neuropathology. Beta amyloid toxicity may occur in part as a result of aggregation of Aβ oligomers [58], and drugs that prevent oligomer aggregation are being tested. Tau proteins are another intriguing target for AD clinical trials, given their pivotal role also in AD pathology. Anti-tau treatments aim to reduce formation of intraneuronal neurofibrillary tangles or to reduce tau phosphorylation. More recently, following the example of immunotherapy directed against beta amyloid, anti-Tau antibodies have begun clinical testing in AD [59]. Anti-inflammatory treatment has also been investigated as a potential therapy for the treatment of AD,

since inflammation occurs at sites of amyloid plaque deposition that may contribute to neuropathology in AD. To date, results of these trials have been disappointing [60, 61], but interest in anti-inflammatory approaches persists [62].

Gene-based therapies are another important component in the attempt to identify new therapies for AD. As described in the following sections, gene therapy has the potential to target degenerating neuronal systems of the brain specifically, over long time periods, and with novel and biologically potent agents such as nervous system growth factors. As a class, neuronal growth factors stimulate the functional state of neurons, preserve and rebuild synapses, and prevent neuronal death.

AD Gene Therapy

Gene therapy approaches have been reported in a number of preclinical models of AD (e.g., [63–81]). Several of these studies report beneficial effects of a candidate gene on AD-related pathology and cognitive performance. One of these approaches, NGF gene therapy, has undergone three clinical trials in AD, including two Phase 1 and a current Phase 2 study. NGF gene therapy in AD was in fact the first trial of gene therapy in the adult nervous system for any neurodegenerative indication. The rationale and current state of this program are described below.

NGF Gene Therapy for Alzheimer's Disease

Discovery and Effects of NGF

Nerve Growth Factor (NGF) was discovered in the 1950s by Rita Levi-Montalcini and Viktor Hamburger. It is one member of the "neurotrophin" family that includes BDNF, neurotrophin-3 (NT-3), and neutrotrophin-4/5 (NT-4/5), molecules that are structurally related. NGF was discovered serendipitously when it unexpectedly elicited exuberant outgrowth of neurites when cocultured with mouse sarcoma cells, and was subsequently identified as an essential factor for the survival and development of peripheral sensory and sympathetic neurons [82, 83]. Its potential role in nervous system degeneration was not appreciated until nearly 35 years later, when groundbreaking studies demonstrated that NGF prevents the death of *adult* basal forebrain cholinergic neurons in rats after injury [84–86] (Fig. 3.2). Shortly thereafter, NGF was also shown to reverse age-related atrophy of basal forebrain cholinergic neurons in rats [87–89]. Extending these findings, NGF also reduced cholinergic neuronal atrophy in a mouse model of Down's syndrome (trisomy 16), wherein an extra copy of the amyloid precursor protein gene is associated with cholinergic degeneration, and improved cognition [90, 91]. Subsequent work extended these findings to the

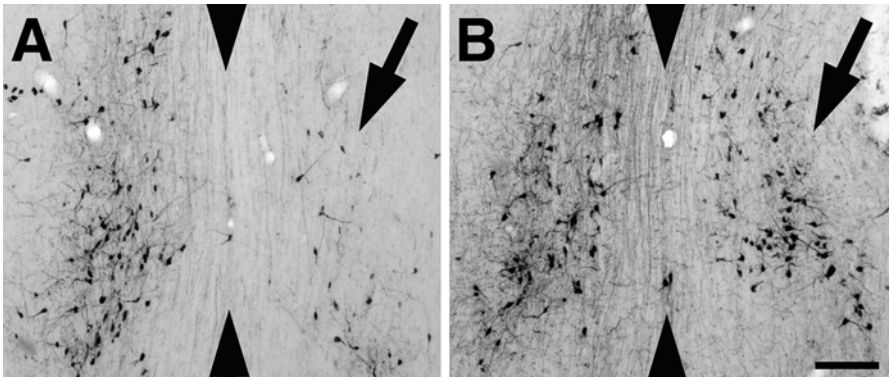

Fig. 3.2 NGF prevents cholinergic neuronal death in rodent models. Intraventricular infusions of NGF protein continuously for 1 month prevent cholinergic neuronal degeneration in adult rats. (**a**) *Right-sided* fornix lesions result in cholinergic neuronal degeneration, reflected by a loss of labeling for the cholinergic neuronal marker choline acetyltransferase on the lesioned, *right side* of the brain compared to the contralateral, intact *left side* of the brain. *Arrowheads* indicate brain midline; *arrows* indicate region of cholinergic neurons affected by fornix lesions. (**b**) NGF gene therapy using injections of cells transduced to secrete NGF substantially reduce cholinergic neuronal degeneration, when assessed 1 month after lesions. Scale bar 250 μm

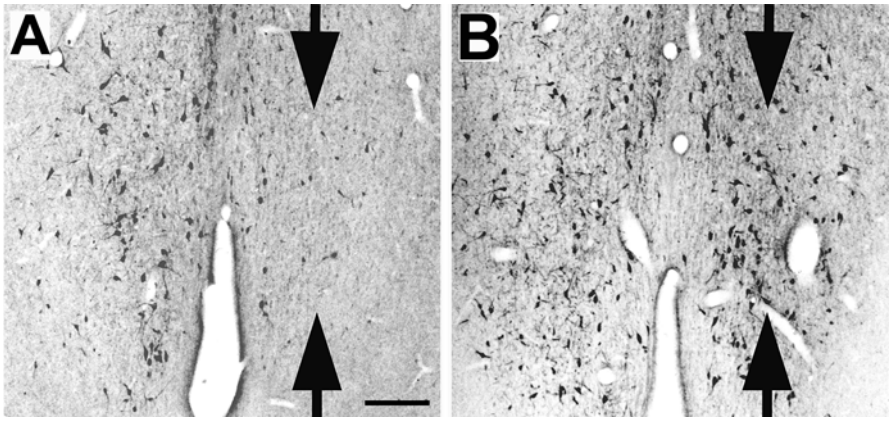

Fig. 3.3 NGF prevents cholinergic neuronal death in nonhuman primates. NGF gene delivery in the rhesus monkey brain prevents cholinergic neuronal degeneration. (**a**) *Right-sided* fornix lesions result in cholinergic neuronal degeneration, reflected by a loss of labeling for the cholinergic neuronal p75 receptor on the lesioned, *right side* of the brain compared to the contralateral, intact *left side* of the brain. *Arrows* indicate region of cholinergic neurons affected by fornix lesions. (**b**) NGF protein infusions for 1 month substantially reduce cholinergic neuronal degeneration after *right-sided* fornix lesions [79]. Scale bar 250 μm

brains of adult lesioned nonhuman primates, and to aged monkeys that exhibit spontaneous, age-related atrophy of cholinergic neurons [65, 72, 92–94] (Fig. 3.3). Furthermore, NGF prevented neuronal degeneration caused by excitotoxic lesions of basal forebrain cholinergic neurons or by cortical lesions in rats [95, 96].

There is a clear rationale for targeting NGF-responsive basal forebrain cholinergic neurons for treatment in AD. Basal forebrain cholinergic neurons of the medial septal nucleus and nucleus basalis of Meynert (NBM) project to neurons throughout hippocampus and neocortex, and modulate neuronal activity. These systems influence attention, memory, and cortical executive functions, and are required for certain complex forms of learning [50, 97–101]. These cholinergic neurons degenerate early in AD [47, 48, 102, 103] likely contributing to symptom development in early and mid-stage AD. Cholinesterase inhibitors partially compensate for reductions in cholinergic systems in AD, demonstrating efficacy in multiple clinical trials in AD [51]. However, these drugs act broadly across brain synapses rather than in a synapse- and time-specific manner that mimics physiological acetylcholine release. Hypothetically, therapies that reduce cholinergic degeneration and sustain normal cholinergic cell function in AD could represent a considerable advance over the use of cholinesterase drugs. Furthermore, dosing of cholinesterase inhibitor drugs in AD is limited by peripheral side effects. While other neuronal systems also degenerate in early AD, the loss of cholinergic neurons represents an important component of the neuropathology. Whether targeting this neuronal system *alone* will be sufficient to slow cognitive decline remains an open question, and a question that the clinical program aims to address.

Thus, basal forebrain cholinergic neurons undergo degeneration in AD and may contribute to characteristic symptoms of the disease. Importantly, these neurons are highly sensitive to NGF, and NGF treatment could represent a means of slowing cholinergic neuronal degeneration in AD. NGF is normally synthesized by cortical and hippocampal neurons, and its availability throughout life sustains cholinergic inputs to these brain regions. Cholinergic axon terminals in the hippocampus and cortex take up the secreted NGF, and retrogradely transport it back to cholinergic cell bodies in the medial septal nucleus and nucleus basalis of Meynert [29]. Upon entry into the cholinergic cell soma, NGF activates the acetylcholine (ACh) synthesizing enzyme, choline acetyltransferase (ChAT), and the vesicular acetylcholine transporter (VAChT) [103, 105]. In AD, NGF levels decline in the basal forebrain while they build up in the cortex, suggesting a retrograde transport defect of NGF to the basal forebrain [29, 31, 106]. The cause of this transport defect is unknown, but could result from alterations in cellular machinery for axonal transport, neuroskeletal instability related to Tau, or perturbations resulting from amyloid precursor protein processing [107]. In theory, NGF directly applied to the cholinergic cell somata in the nucleus basalis of Meynert could bypass NGF transport deficits to sustain and functionally activate [108] the neurons in AD. Receptors for NGF are expressed on both cholinergic axons and cell bodies.

The preceding considerations led to an effort to treat AD patients with NGF protein infusions approximately 20 years ago [108]. Because NGF is a medium-sized, moderately polar molecule that does not cross the blood–brain barrier, early animal studies infused it into the lateral ventricles of the brain [84, 86, 109]. This route of administration succeeded in preventing cholinergic neuronal death and stimulating cell function, but it also broadly spread NGF through the cerebrospinal fluid. As a result, several off-target effects occurred in animal models, including weight loss,

sensory (nociceptive) and sympathetic axon sprouting that caused pain-related behaviors, and proliferation of Schwann cells around the medulla and spinal cortex [110–113]. Of three AD patients who received NGF protein infusions into the cerebral ventricles, all three reported dull and constant back pain and two exhibited weight loss during NGF infusion [108]. Thus, despite the potential promise of NGF therapy in AD, these side effects required the development of new and better targeted means of NGF administration.

NGF Gene Therapy: Phase I Ex Vivo Clinical Trial

Following the initial failure of protein intracerebroventricular infusions, methods for delivering NGF were sought that could provide adequate protein concentrations in *targeted* brain regions (the nucleus basalis of Meynert) to prevent cholinergic neuronal degeneration, while *restricting* NGF distribution *solely* to this brain region to avoid off-target adverse effects. A potential solution to this problem was gene therapy. The physical size of the nucleus basalis of Meynert in humans is approximately 1 cm in length, and targeting this structure could impact cholinergic axon terminals throughout the cortex. Thus, the nucleus basalis of Meynert seemed a practical target that could be accessed in humans by neurosurgeons, using methods of gene delivery.

The brain consists nearly exclusively of nondividing cells, and at the time that the first efforts were being made to translate NGF gene therapy to humans in the 1990s, gene therapy vectors for targeting nondividing cells of the brain were unsatisfactory. Adeno-associated viral vectors were not yet in extensive clinical use, and adenoviral vectors expressed wild-type genes that could generate immune responses. Thus, we pursued ex vivo gene transfer methods for NGF gene delivery in AD. Patient autologous fibroblasts were genetically modified to produce and secrete human NGF using Moloney leukemia virus (MLV) retroviruses containing the relatively robust LTR (long terminal repeat) promoter. In preclinical experiments in rats and primates, this delivery method was found to deliver NGF to the basal forebrain cholinergic nuclei without extensive spread to other brain regions [65, 73, 77, 80]. Moreover, ex vivo NGF gene delivery was effective in significantly reducing lesion-induced cholinergic neuronal degeneration in both rodents and primates [65, 73, 77, 80]. Importantly, NGF did not detectably spread to other brain regions, and the off-target effects of intracerebroventricular NGF protein infusions did not occur. Demonstrations of NGF efficacy after gene transfer were extended to studies in aged rats, where age-related cholinergic cell atrophy was reversed and cognitive function improved [114]. Further studies in aged monkeys, performed in a manner to mimic potential human clinical trials, demonstrated that autologous fibroblasts expressing NGF reversed age-related neuronal atrophy restored cholinergic axon numbers in the neocortex [115, 116]. Expression of NGF persisted for at least 1 year. Dose escalation and safety-toxicity studies demonstrated no adverse effects in monkeys. Taken together, these preclinical experiments indicated that ex vivo NGF

gene therapy could accurately deliver NGF to the basal forebrain without eliciting adverse effects. Accordingly, the program advanced to human clinical trials.

The first human trial of gene therapy in an adult neurodegenerative disorder began in 2001. Eight patients with early-stage AD were enrolled into a Phase 1 trial of ex vivo NGF gene therapy [117]. This clinical program aimed, through this initial and subsequent phase trials, to test the hypothesis that NGF could reduce cholinergic neuronal degeneration and stimulate neuronal function in AD, thereby detectably modifying disease progression. This first phase 1 trial had more modest objectives, aiming to determine whether NGF gene delivery could be accomplished safely in AD patients. Primary autologous fibroblasts were taken by skin biopsies from each patient and expanded in a good manufacturing process (GMP) facility. They were then transduced with Moloney murine leukemia virus (MLV) vectors to express recombinant human full-length NGF. The NGF gene therapy vector included the pre-pro form of the NGF coding sequence and allowed packing of NGF into Golgi apparatus and subsequent secretion from the cell. Indeed, high levels of NGF were produced, generally on the order of 100 ng/10^6 cells/day, and most (99 %) of this NGF was secreted from the fibroblasts into the cell culture medium, providing a means of "bathing" the local cellular environment with NGF. In the adult brain, most NGF secreted from cells undergoes proteolytic cleavage to its active, pro-survival form; however, NGF can also exist extracellularly in its pro-form, which can promote cellular apoptosis [118]. NGF secreted from our transduced fibroblasts was nearly entirely the proteolytically cleaved, mature form of NGF, lacking pro-NGF. Clinical stereotaxic frames were used to implant patients' NGF-secreting autologous cells adjacent to the cholinergic basal forebrain. The first two patients received implants of NGF-secreting cells solely into the right side of the brain, and the subsequent six patients received bilateral cell implantations. A range of cell "doses" (total numbers) was administered, from 1.25 million cells implanted unilaterally to 5 million cells implanted bilaterally.

Subjects were monitored posttreatment for the remainder of life. Adverse effects of NGF expression in the brain were not detected: pain, weight loss, or Schwann cell migration did not occur. An intraparenchymal hemorrhage occurred in one patient during the NGF gene transfer procedure that contributed to the patient's death approximately 1 month later. The future risk of this complication was addressed by modifying the design of the brain injection needle, and in 60 patients subsequently treated in phase 1 and phase 2 clinical trials, no additional hemorrhages have been reported.

Statements regarding efficacy could not be made from the first phase 1 ex vivo NGF gene therapy safety trial because only eight patients were enrolled, there was no control group, and blinded assessments could not be made. But cognitive function was measured serially over time and compared to preoperative baselines on the Mini-Mental Status Examination (MMSE) and Alzheimer's Disease Assessment Scale-Cognitive component (ADAS-Cog) [117]. Cognitive testing performed over a 2-year period following gene transfer showed an approximate 50 % reduction in the rate of cognitive decline after gene transfer compared to testing performed prior to the surgery. However, no conclusions regarding efficacy could or should be drawn

from these data given the small sample size, lack of control group, and non-blinded nature of data collection. One could reasonably conclude that, in this small set of patients, gene transfer did not appear to cause a worsening of cognitive function. Four bilaterally treated subjects who underwent NGF gene transfer were also examined by serial PET scans prior to and after treatment. Comparing post- to pretreatment 18-Fluorodeoxyglucose uptake, a significant ($P<0.05$) *increase* in cortical uptake was found after gene transfer, using a systems, region-of-interest analysis (Fig. 3.4) [117]. These findings counter the typical AD pattern of loss of 18-Fluorodeoxyglucose uptake over time, although the sample size is too small to draw definitive conclusions.

Postmortem pathological analysis was performed in all subjects in the first phase 1 trial, up to 10 years after gene transfer [119]. All subjects at the time of death had advanced Braak stage V–VI Alzheimer's disease. Surviving fibroblasts were observed in all cases in the region of NGF gene transfer. Most notably, every brain exhibited evidence of a trophic response to NGF, reflected by cholinergic axon sprouting into the fibroblast graft (Fig. 3.5). These findings indicate that degenerating neurons in the AD brain are consistently able to exhibit a classic "trophic" (sprouting) response to NGF. Sub-analysis of brains in subjects who underwent unilateral gene transfer demonstrated cell hypertrophy of ~8 % on the treated side ($P<0.05$), another trophic response.

In summary, the first Phase 1 clinical trial of ex vivo NGF gene therapy in AD supported the rationale of providing trophic support to cholinergic neurons, a program that has been expanded into a phase 2 program.

NGF Gene Therapy: Phase 1 and 2 In Vivo Clinical Trials

The NGF ex vivo gene therapy clinical program began before in vivo gene therapy vectors were widely available for clinical use. However, by the completion of the Phase 1 ex vivo trial, experience with adeno-associated virus (AAV) vectors had advanced considerably. Clinical trials with in vivo gene therapy vectors offered substantial potential advantages. In ex vivo gene therapy trials, patient fibroblasts were biopsied, expanded, transduced to express the gene of interest, tested, harvested, and shipped to a physician for intracranial injection. In vivo gene therapy is substantially simpler: the AAV vector product can be processed in large batches, characterized, tested, and sent to a physician for injection. Moreover, in preclinical studies, AAV2-NGF gene delivery was as safe and effective compared as ex vivo gene therapy [120]. In addition, the duration of in vivo gene expression was superior when using AAV2 vectors [121]. For these reasons, a decision was made to switch NGF program development from ex vivo gene therapy using grafts of autologous fibroblasts to in vivo gene therapy using AAV2-NGF vectors. Several other clinical trials also utilized AAV gene therapy in the nervous system [122–125] (see Chaps. 2, 4, 5, and 7).

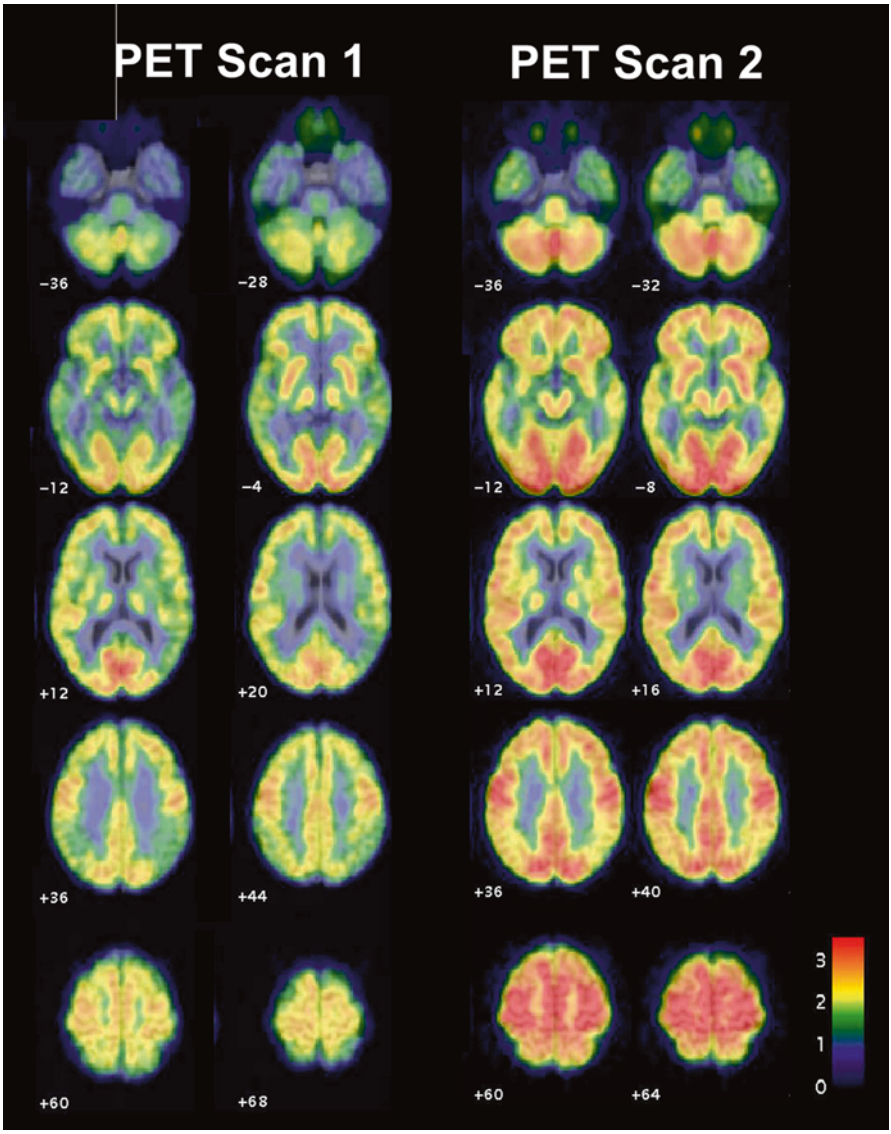

Fig. 3.4 PET scans in patients undergoing NGF gene therapy. Glucose uptake by PET scan in subjects who underwent ex vivo NGF gene therapy, overlaid on standardized MRI templates. Images are average PET data from all four bilaterally treated patients who underwent serial PET scans. Representative axial sections, with 6–8 months between first and second scan, show interval increases in brain metabolism in diverse cortical regions, representing the broad cortical projection pattern of cholinergic systems. Flame scale indicates FDG use per 100 g tissue/min; *red color* indicates more FDG use than *blue* (from [117])

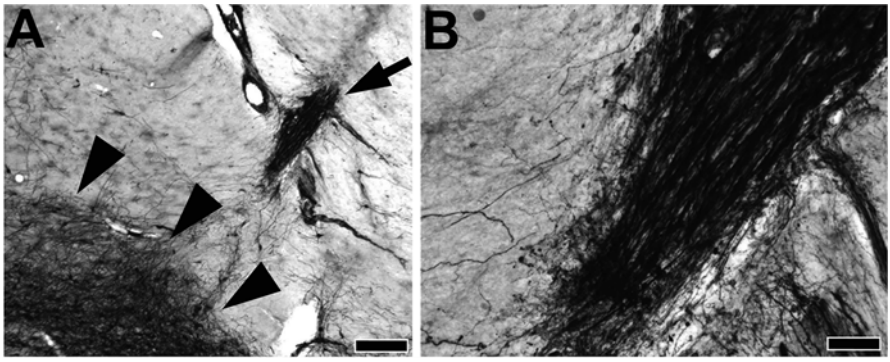

Fig. 3.5 Cholinergic axon trophic responses in humans undergoing NGF gene therapy. P75 label for NGF-sensitive cholinergic neurons. (**a**) Human brain 7 years after ongoing NGF gene therapy shows surviving graft of autologous fibroblasts penetrated by host cholinergic neurons (*arrow*) located adjacent to region of cholinergic neuronal cell bodies in the Nucleus Basalis of Meynert (*arrowheads*). (**b**) NGF secreting cell graft at higher magnification, exhibiting dense penetration by cholinergic axons. Scale bar (**a**), 500 μm; (**b**), 50 μm

Preclinical studies using AAV-NGF gene delivery supported the transition to clinical trials. AAV2-NGF gene therapy ameliorated age-related deficits in spatial memory and resulted in increases in cholinergic cell size in aged rats [126]. AAV2-NGF was also shown to be neuroprotective in the brains of aged rats and to sustain gene expression over time [120]. Another in vivo gene therapy vector, lentivirus (expressing NGF), also prevented the death of cholinergic forebrain neurons following fimbria fornix lesions in rats [127]. In the primate brain, Lenti-NGF resulted in long-term in vivo NGF expression and reversed age-related declines in the number and size of basal forebrain cholinergic neurons [128].

Supported by these preclinical data, a company-sponsored Phase 1 clinical trial of AAV2-NGF was initiated in nine patients. Subjects had mild to moderate AD (mini-mental status exam scores of 16–28) [129]. AAV2-NGF was stereotaxically injected in the nucleus basalis region at one of three doses. All AAV2-NGF doses were found to be safe and well tolerated, and this program progressed to initiation of a Phase 2 multicenter trial primarily funded by the National Institutes for Aging in 2011.

We received brains from two patients who received AAV2-NGF in the preceding clinical trial (Fig. 3.6). NGF was persistently expressed by cells in the nucleus basalis of Meynert at 11 months and 3 years posttreatment, respectively (Fig. 3.6). NGF appeared to induce cholinergic neuronal hypertrophy, and in both subjects appeared to increase expression of the immediate early gene c-fos in the zone of NGF expression.

The phase 2 trial of AAV2-NGF gene delivery in AD is designed to extend the safety and tolerability data of AAV2-NGF gene delivery program in AD, and to determine the potential effect size of AAV2-NGF in AD patients. The trial is a double-blind design, and includes a sham surgery control group. A total of 49

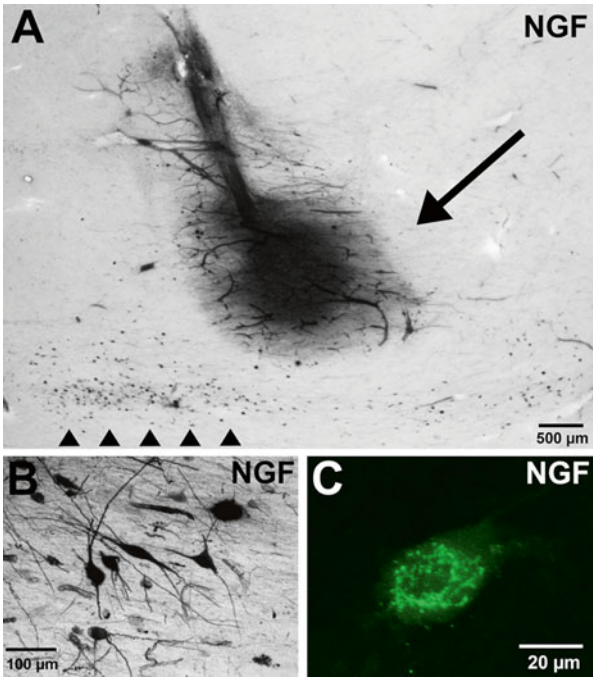

Fig. 3.6 NGF expression and spread in AAV2-NGF gene therapy trial. NGF immunolabeling of cells in the cholinergic basal forebrain of AD patients who underwent AAV2-NGF gene transfer, (**a–b**) 1 year earlier, and (**c**) 3 years earlier. (**a**) NGF immunoreactivity at the injection site (*arrow*); cells of the nucleus basalis of Meynert are indicated by *arrowheads*. (**b**) Higher magnification image of NGF-immunolabeled cells with typical neuronal morphology. (**c**) Fluorescent NGF labeling shows a granular pattern of NGF labeling within the cell soma, likely reflecting packaging of NGF in the endoplasmic reticulum/Golgi apparatus and sorting into dense-core vesicles [173]

patients are enrolled at 1 of 11 clinical centers across the United States [130]. Approximately half of patients receive the highest AAV2-NGF "dose" of the phase 1 trial (2×10^{11} viral genomes per brain). The other half of patients undergo sham surgery that involves drilling a partial burr hole, without penetrating the dura. The primary endpoint is change in the Alzheimer's Disease Assessment Scale-Cognitive subcomponent (ADAS-Cog) over a 2-year follow-up period. Several secondary endpoints are also being measured, sampling several cognitive realms. This Phase 2 trial is underpowered to yield a statistically significant determination of AAV2-NGF treatment on cognitive function, but it is expected to provide an estimate of the potential "effect size" of AAV2 NGF, allowing a "go" versus "no-go" decision for this clinical program, using its current gene delivery methods (see below). If a sufficient potential efficacy signal is detected, then an adequately powered subsequent efficacy trial will be designed.

A challenge that has become evident in histopathological analysis of brains of patients treated in the Phase 1 ex vivo and Phase 1 in vivo NGF therapy trials is the accuracy of neurosurgical targeting of deep brain structures with vector delivery.

At the AAV2 vector concentrations and volumes that have been administered in these clinical programs, NGF spread from the injection site is generally less than 2 mm. In several brains identified to date, NGF expression may not have reached the targeted cholinergic neurons, a caveat that will need to be considered in interpreting the outcomes of the Phase 2 AAV2-NGF trial. Improved methods of vector targeting may be needed. Such methods are being integrated into the AAV2-BDNF program in AD described below, including real-time MR imaging of vector administration and convection-enhanced delivery of larger vector volumes to enhance vector spread.

The Phase 1 clinical trial of ex vivo gene therapy began 14 years ago. With results of the Phase 2 trial expected soon, the program is at a pivotal point. One might reflect back on the long length of time it has taken to come to this point. The slow pace of progression of this program likely is due to several factors: (1) caution of the gene therapy clinical trial realm when the program was initiated in the early 2000s, and some continued caution today; (2) the challenges of funding an early-stage clinical program that breaks new ground using novel technologies; and (3) the difficulty of conducting clinical trials in complex neurodegenerative disorders that largely lack robust clinical measures and efficacy end points. These challenges are not unique to the field of gene therapy or to Alzheimer's disease.

BDNF Gene Therapy for Alzheimer's Disease

Rationale

BDNF is another neurotrophic factor with substantial potential to reduce neuronal degeneration and stimulate neuronal function in cell systems that degenerate in AD. BDNF, like NGF, supports neuronal survival, axonal outgrowth, and appropriate target innervation during neural development. In contrast to NGF, which influences cholinergic neurons of the basal forebrain, BDNF directly influences the survival and function of cortical and hippocampal neurons [131, 132]. Neurons of the cortex and hippocampus are central to the development of cardinal symptoms of AD, and these same neurons express the BDNF receptor, trkB [131, 132]. In the NGF gene therapy program, NGF could prevent or slow the loss of cholinergic neurons of the basal forebrain, thereby improving symptoms in early and middle stages of the disease. However, NGF does not directly support neurons of the cortex and hippocampus, and these neurons would eventually degenerate even if NGF were successful in preserving cholinergic systems. In contrast, BDNF gene therapy could in theory directly preserve or prolong the survival of key hippocampal and cortical circuitry that degenerates in AD, providing a more potent or sustained effect.

During life, BDNF functions as an important molecule in normal brain function. BDNF influences gene expression, and, at the level of synapses, vesicular clustering and docking, neurotransmitter release, and long-term potentiation [133, 134].

A key focus of the BDNF gene therapy program in AD is the entorhinal cortex. This medial temporal lobe structure exerts a critical role in new memory formation [135]. Diverse brain regions project into the entorhinal cortex, which acts as a relay to hippocampal circuitry. The hippocampus then projects back to the entorhinal cortex, which in turn projects to the diverse cortical regions that represent storage sites of many types of memories. Collectively, new memory formation requires essential cellular and electrophysiolgical "plastic" mechanisms contained within hippocampal and entorhinal circuits.

Notably, one of the earliest brain regions affected by AD pathology is the entorhinal cortex [19, 38, 40]. A direct consequence of degeneration of entorhinal–hippocampal circuits is the earliest and cardinal clinical symptom of AD, short-term memory loss [14, 16]. The spread of neuropathology in AD may reflect anatomical connections of other brain regions with the entorhinal cortex, as a consequence of either spreading beta amyloid [20, 43, 44] or Tau [45, 46] pathology. Significantly, levels of BDNF decline in the entorhinal cortex in the earliest stages of AD [28, 30, 106]. Thus, it is hypothetically possible that specific targeting of BDNF to entorhinal circuitry could dampen early pathogenesis in AD, thereby delaying progression of short-term memory loss. If disease pathologically is directly propagated from the entorhinal cortex to other brain regions, it is also possible that BDNF-mediated neuroprotection in the entorhinal cortex could delay disease progression to other brain regions. Accordingly, in preclinical models of AD we investigated whether BDNF gene therapy to entorhinal circuitry prevented neuronal degeneration and stimulated neuronal function [75, 136].

Preclinical Studies of BDNF Gene Therapy

We examined six models of AD to determine whether BDNF exerted neuroprotective effects in entorhinal/hippocampal circuitry [75]. Transgenic mice that overexpress known human mutations in the amyloid precursor protein (APP) gene are one of the most broadly studied models of AD [137]. Humans bearing these mutations suffer a premature onset of AD. We injected lentiviral vectors expressing BDNF into the entorhinal cortices of APP transgenic mice at 6 months of age, *after* disease onset; mice express characteristic plaque deposition and the beginning of neuronal loss prior to this age [75, 136]. The BDNF gene was expressed in the entorhinal cortex, but the locally produced BDNF protein spread by anterograde transport down axons and into the hippocampus (Fig. 3.7a). BDNF protein in the entorhinal cortex and hippocampus was associated with a reversal of loss of the presynaptic protein synaptophysin. BDNF gene delivery also activated neuronal ERK, an important cell signaling molecule related to functional state of neuronal activation. Transcriptome analysis showed that BDNF treatment reversed two-thirds of APP-induced disruptions in global gene expression caused by the APP mutation (Fig. 3.7b). Moreover, APP-transgenic mice treated with BDNF exhibited improvement on two cognitive measures of hippocampal-dependent learning and memory:

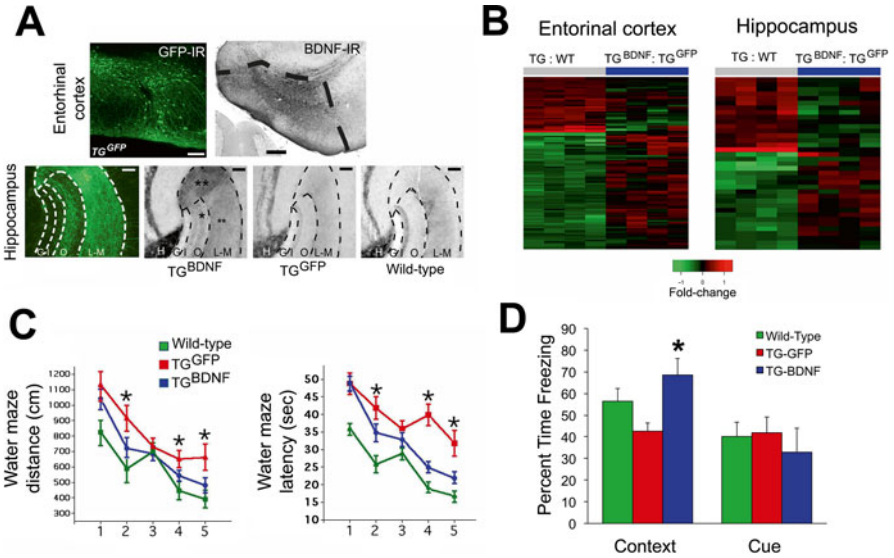

Fig. 3.7 BDNF gene therapy in APP transgenic mice. BDNF effects in APP-transgenic mice. (**a**–*Upper panel*) Lentiviral gene delivery results in efficient gene expression (*left*: GFP reporter) and dense BDNF immunolabeling (*right:* dark labeling) in the entorhinal cortex of amyloid transgenic mice that received injections of BDNF-expressing vector. (**a**–*Lower panel*) Axon terminals of entorhinal cortex neurons projecting into the hippocampus are identified after entorhinal lenti-GFP injection in APP-transgenic mice (*left panel*, TG^{GFP}); the same pattern of BDNF expression is observed in TG^{BDNF} mice, indicating anterograde transport of BDNF into CA stratum lacunosum-moleculare (L-M) (*double asterisk*) and dentate outer molecular layer (O, *asterisk*), compared to TG^{GFP} controls and WT mice. *H* hilar region, *G* granule cell layer, *I* inner molecular layer. (**b**) Heat maps depicting fold changes of APP-related genes before and after treatment with BDNF. 107 probe sets are differentially expressed in entorhinal cortex of TG mice compared to WT (TG:WT), and 37 probe sets are differentially expressed in hippocampus (*gray bar columns*, $P<0.005$ by Bayesian *t*-test). (**c**) *BDNF* gene delivery to the entorhinal cortex improves spatial memory in the Morris water maze in mutant APP-transgenic mice (TG) on distance (*left*) and latency (*right*) measures ($P<0.005$ by ANOVA, $^*P<0.05$ by post hoc Fisher's test, comparing TG^{BDNF} to TG^{GFP}). (**d**) BDNF significantly improves hippocampal-dependent (hpc.-dep.) contextual fear conditioning ($P<0.05$); no deficits were observed in the TG^{GFP} compared to the WT control. Scale bar (**a**) *upper panel*, *left* = 100 μm, *upper panel*, *right* = 200 μm, *lower panel* = 50 μm

the Morris water maze and contextual fear conditioning (Fig. 3.7c, d). However, BDNF gene delivery had no effect on amyloid plaque deposition, suggesting that beneficial effects of BDNF gene delivery were independent of reductions in amyloid plaque density in the brain. Instead, BDNF appeared to exert direct effects on neuronal survival, synaptic proteins, and gene expression.

More recently, we initiated treatment with BDNF at an earlier time point in APP transgenic mice, before disease onset [136]. The purpose of this study was to determine whether BDNF treatment could prevent neuronal loss in the entorhinal cortex that proceeds over several months in APP transgenic rats. Indeed, when treatment was initiated at 2–3 months of age, neuronal loss was significantly reduced when

examined at 7 months of age. In addition, early life BDNF gene delivery produced a significant improvement in learning and memory, and an increase in the presynaptic protein synaptophysin in the entorhinal cortex and hippocampus [136]. Once again, BDNF did not affect amyloid plaque load, suggesting that BDNF acts through mechanisms independent of amyloid.

Another model of AD, albeit imperfect, is normal aging. Aging is a major risk factor for the development of AD, and aging is associated with neuronal atrophy and declines in several functional cellular markers. We infused recombinant BDNF protein into entorhinal cortices of aged Fischer 344 rats. When examined 2 months later, we found that BDNF infusions ameliorated age-related declines in spatial memory, restored levels of ERK phosphorylation to levels of young animals, and reversed age-related perturbations in gene expression [75].

We then extended these studies to nonhuman primates. Aged rhesus monkeys were tested for baseline function on a computerized memory task and then underwent lentiviral BDNF gene delivery to bilateral entorhinal cortices. We found that BDNF gene delivery ameliorated age-related impairments in short-term memory, and subsequent anatomical analysis showed induction of entorhinal neuronal hypertrophy. Thus, BDNF gene delivery to the entorhinal cortex of nonhuman primates exhibited significant effects on cognition and cell morphology [136].

We also examined whether BDNF gene delivery to the entorhinal cortex could prevent another mechanism of cell dysfunction: axotomy-induced neuronal degeneration [136]. Rats underwent perforant pathway lesions that sever axonal projections from the entorhinal cortex to the hippocampus. In lesioned subjects lacking BDNF treatment, a significant loss of neurons occurred in layer II of the entorhinal cortex. In contrast, animals that received BDNF gene delivery showed significant reductions in cell death [75]. Extending these findings once again to a nonhuman primate model, we found that perforant pathway lesions in adult monkeys caused nearly 50 % loss of neurons in layer II of the lateral entorhinal cortex and that BDNF gene delivery rescued the majority of these cells from death [75] (Fig. 3.8a, b). Thus, BDNF demonstrated neuroprotection across species, animal models, and mechanisms of experimental neurodegeneration.

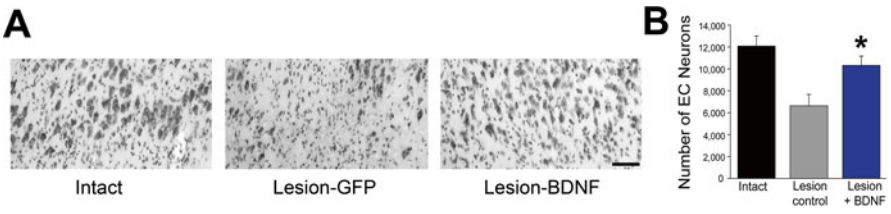

Fig. 3.8 BDNF gene therapy in lesioned monkeys: prevention of entorhinal neuronal death. (**a**) Nissl-stained sections of layer II in entorhinal cortex in intact monkey, in a monkey with a perforant path lesion and Lenti-GFP treatment, and in a monkey with a perforant path lesion and Lenti-BDNF treatment. Scale bar 65 μm. (**b**) Stereological analyses showed a significant loss of cells in layer II entorhinal cortex following a perforant path lesion in control lesioned monkeys, and a significant amelioration of cell loss after BDNF gene delivery (*$P<0.01$ vs. lesion control)

Collectively, the preceding studies support the hypothesis that BDNF can promote neuronal survival and augment the functional state of neurons in correlative models of AD. These findings are supported by other studies. Another group demonstrated that BDNF gene delivery into the brains of APP transgenic mice also improved synaptic markers and memory performance, without directly affecting beta amyloid [70]. In separate studies, gene delivery of a binding protein for CREB (cAMP-response element binding protein) increased BDNF expression and improved memory deficits in an AD mouse model [138]. Furthermore, neural stem cells secrete BDNF, and transplantation of neural stem cells in a transgenic mouse model of AD improved synaptic markers and improved memory; these improvements were not observed when BDNF expression was blocked in the transplanted cells [137].

Given the preceding body of evidence across disease models and laboratories, we propose to develop AAV2-BDNF gene therapy for clinical trials. Like NGF, broad distribution of BDNF in the central nervous system has the potential to generate off-target effects that could be disabling. Nervous system growth factors must be administered to regions of neurodegeneration without spread to non-degenerating regions to avoid these potential adverse effects. Two potential paths present themselves for this clinical program: gene therapy or intraparenchymal protein infusion. Given the relative simplicity of gene delivery and its track record of safety in hundreds of patients enrolled across several trials [117, 122–124, 140–142], we are focusing for the present on gene delivery.

Improved Tools for Gene Delivery to the Brain: Real-Time Imaging and Convection Enhanced Delivery of AAV2-BDNF in AD

Targeting deep structures for gene delivery in the human brain is a challenge. Stereotaxic neurosurgical procedures have a resolution in experienced hands of 1–2 mm in accurately reaching their target, but this may not be sufficiently accurate when targeting a structure like the nucleus basalis of Meynert in the NGF program, or the entorhinal cortex in the BDNF program. For example, the thickness of the human entorhinal cortex from cortical surface to subcortical white matter is approximately 3 mm [143, 144], and the preferred target is layer II–III of the entorhinal cortex, which is ~1 mm thick. Ideally, a method would be available in which (1) a catheter for gene therapy vector infusion into the brain can be imaged in real time and the accuracy of its position confirmed, and (2) spread of vector through the intended targeted brain region can be confirmed in real time. These methods have recently been developed by Bankiewicz and colleagues [145–148]. Using MRI-compatible ceramic infusion needles and co-infusion of gene therapy vector with a radiological tracer (gadoteridol), they report an ability to accurately target AAV vector delivery to brain structures [149, 150]. The addition of convection-enhanced delivery techniques can further optimize vector distribution in a desired brain target region [150, 151]. Convection-enhanced delivery is an infusion technique wherein

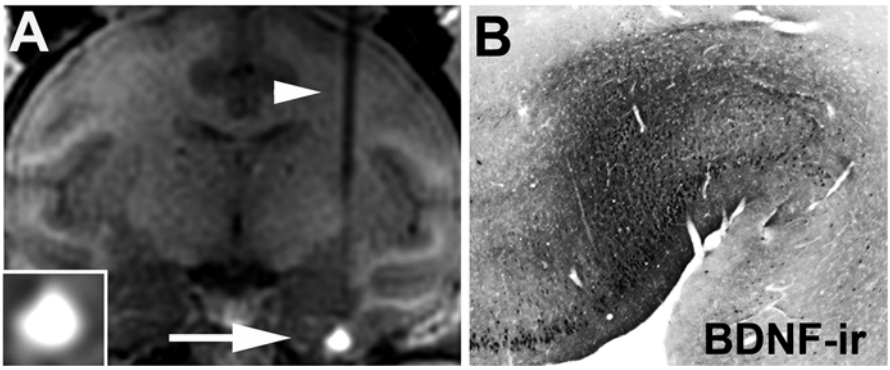

Fig. 3.9 Real-time AAV2-BDNF vector delivery to the nonhuman primate. (**a**) MRI scan showing infusion of AAV2-BDNF into rhesus monkey brain. *Arrow* indicates infusion of AAV2-BDNF/gadoteridol into entorhinal cortex; *arrowhead* indicates MRI-compatible injection needle. (**b**) BDNF immunoreactivity in the entorhinal cortex confirms AAV2-BDNF infusion, with resultant expression of BDNF protein by immunolabeling that corresponds to spread of gadoteridol by MRI

continuous flow rates that exceed intraparenychmal hydrostatic pressure (0.5 μl/min for the brain) result in a "push" of fluid more extensively through the brain (Fig. 3.9). These technological developments could represent a major breakthrough for targeting and distribution of gene therapy vectors in human neurological disorders. Previous "blind" methods of gene delivery using standard stereotaxic infusions have resulted in vector distribution more limited in distribution than had been modeled in preclinical studies [152], highlighting the need for improvements in delivery technology. We have developed these procedures to target the entorhinal cortex in nonhuman primates (Fig. 3.9), and will implement this technology in clinical trials of AAV2-BDNF in AD.

Gene Therapy in AD: Delivery of Other Therapeutic Genes

Alterations in amyloid processing and deposition represent a major target in AD drug development. Several studies have used techniques of gene therapy to target beta amyloid in animal models of AD, as described below [66, 67, 74, 78, 81, 153]. Gene therapy methods have also been employed to target other AD-related mechanisms, including tau and neuroinflammation.

Gene Therapy and Amyloid Degradation

Gene therapy has been used in preclinical studies to degrade amyloid in mice overexpressing human amyloid mutations. For example, overexpression of the enzymes neprilysin (NEP), insulin-degrading enzyme (IDE), or endothelin-converting enzyme (ECE) can modify amyloid accumulation in the brains of transgenic mice.

Neprilysin, a natural brain enzyme, is a membrane-bound metalloprotease that degrades several small peptides including beta amyloid [154]. In AD, levels of Neprilysin mRNA and protein are reduced [155, 156]. When experimentally overexpressed in the brain of APP transgenic mice, neprilysin, insulin-degrading enzyme, or endothelin-converting enzyme all reduce amyloid plaque formation [64, 78, 157, 158]. Masliah and colleagues also reported that systemic delivery of a gene therapy vector could achieve reduction in brain amyloid [159]. They used a lentiviral vector expressing neprilysin fused with Apoliproprotein B (ApoB), a low-density lipoprotein receptor-binding domain that is able to cross the blood–brain barrier. When administered to APP transgenic mice, neprilysin crossed the blood–brain barrier and reportedly improved synaptic density and memory performance [159]. An independent study also reported that invascular injection AAV9-Neprilysin elevated neprilysin activity in the brain and reduced amyloid oligomers [160]. Thus, gene therapy to express enzymes that degrade beta amyloid represents a potential therapy for AD.

As summarized above, a number of AD clinical trials attempt to autoimmunize patients against beta amyloid to enhance its clearance. Gene therapy has been reported as an alternative means of expressing peptide fragments in the brain that could result in immunization. For example, AAV viral vectors encoding synthetic beta amyloid fragments result in production of amyloid antibodies in a mouse model of AD when combined with an adjuvant (granulocyte/macrophage-colony stimulating factor) [161]. Amyloid antibodies were formed and the density of plaques was reduced in transgenic mice [66, 162].

RNA Interference in AD

Gene therapy can also be used to produce small interfering RNAs (siRNAs) in the brain. For the treatment of AD, siRNAs that suppress beta or gamma secretase expression could reduce amyloid precursor protein cleavage at sites leading to beta amyloid production. In one study, lentiviral vectors expressing siRNAs to beta secretase and infused into the hippocampus significantly reduced amyloid deposition and improved cognitive in APP transgenic mice [153]. Gene delivery of siRNAs directed against presenilin 1, a protein that elevates beta amyloid levels, can also reduce beta amyloid in mouse models of AD [163]. RNA interference has also been directed against tau, using siRNAs that reduce levels of GSK-3 and CDK5 to reduce tau phosphorylation [76, 164].

Other Gene Therapy in AD

AAV-mediated gene delivery of interleukin-10 (IL-10) or interleukin-4 (IL-4) appears to reduce astrogliosis and microglial activation, resulting in reduced amyloid accumulation in amyloid mutant mice [72, 165]. Overexpression of a CREB binding protein in mice expressing mutant amyloid increased BDNF protein levels

and improved memory, as noted above [138]. Other gene delivery studies have also reported therapeutic effects in animal models of AD [76, 166, 168]. Thus, a broad range of possibilities for future gene-based therapy of AD exist.

Future Gene Therapy for AD

A key to the future development of gene therapy for any indication, including AD, is the ability to regulate the expression of viral vector products [127, 168]. This is especially important when a candidate gene delivery product could have adverse consequences, as in off-target effects of neurotrophic factors. While more than 200 humans treated in AD and Parkinson's disease programs to date have shown no evidence of off-target effects from trophic factor overexpression, regulatable expression or incorporation of a suicide gene in the expression vector would enhance the safety profile of gene therapy. Another active focus of development in the gene therapy field is the design of novel vectors, hybrid vectors, and cell-specific promoters that enhance neuron or glia-specific gene expression, movement across the blood–brain barrier, and retrograde vector transport [169–172] (see Chap. 2).

Summary

Gene delivery is an intriguing tool for the potential therapy of AD and other disorders. It solves the problem of achieving regionally specific delivery of large proteins into the brain. Modern vectors sustain gene expression over prolonged time periods—years—after a single vector administration procedure, providing the possibility for prolonged in vivo efficacy and eliminating the need for daily drug ingestion. Yet a breakthrough has not yet come, and carefully designed and conducted clinical trials remain necessary to ultimately prove the value of this technology for human disease treatment. Promising possibilities from the field of nervous system disorders, ocular diseases, inborn errors of metabolism, or cancer could provide this breakthrough.

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Chapter 4
GDNF and AADC Gene Therapy for Parkinson's Disease

Krystof Bankiewicz, Waldy San Sebastian, Lluis Samaranch, and John Forsayeth

Abstract The use of viral vectors to express therapeutics genes in Parkinson's disease (PD) trials has been hindered by a lack of understanding of the principles that guide effective distribution of vectors within the basal ganglia, even when we have a strong expectation of efficacy based on experimentation in animal models. The major problems we have faced include (1) scale-up from small rodent and nonhuman primates (NHP) brains to humans, (2) understanding how viral vectors distribute within the brain parenchyma, (3) prediction of how viral particles are disseminated by neuronal projections after direct delivery to the putamen and substantia nigra, (4) the mechanism of action of the therapeutic gene on dopaminergic system, and (5) the relevance of animal models to idiopathic PD. In this chapter, we will address these important issues and will try to put them into the context of data that has been obtained from current and recent clinical trials. In particular, we will address therapeutic strategies aimed at restoring dopaminergic function by either expressing genes that encode enzymes responsible for synthesis of dopamine (DA) or expressing growth factors capable of upregulating DA function in the degenerated neurons. We will not address inhibition of outflow innervation from the striatum in PD patients by expressing glutamic acid decarboxylase (GAD) as this strategy is described in the dedicated chapter in this book.

Keywords Adeno-associated viral vector • MRI-guided brain delivery • Convection-enhanced delivery • Aromatic L-amino acid decarboxylase • Neurotrophic factor • Clinical trial

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Introduction

PD is a common, mostly idiopathic, neurodegenerative disease characterized by resting tremor, rigidity, bradykinesia, and postural instability. It has an estimated incidence of 1–2 cases per 1000 in the general population, and 2 cases per 100 among people older than 65 years [1]. Pathologic features include the loss of DA neurons in the substantia nigra (SN), locus ceruleus, globus pallidus, and putamen, with gliosis, eosinophilic neuronal cytoplasmic inclusions (Lewy bodies), and proteinaceous inclusions in neurites (Lewy neurites) within the basal ganglia, brainstem, spinal cord, and sympathetic ganglia. However, it should be borne in mind that PD is also a systemic disease that attacks peripheral catecholaminergic neurons in the gut and heart earlier than the central deficits [2]. Disease severity generally correlates with the degree of DA deficiency; however, clinical presentation of the motor deficit tends to be late in the pathological process, after an estimated 70–80 % loss of putaminal innervation by projections from the SN pars compacta [3].

Standard PD therapy is primarily symptomatic and pharmacologic, involving DA replacement (levodopa), DA receptor agonists (pramipexole and ropinirole), potentiators of DA release (amantadine), and agents that slow the breakdown of DA in the synaptic cleft (monoamine oxidase inhibitors). Levodopa, the biosynthetic precursor of dopamine, is the most commonly used and most effective of these treatments. It is orally administered, crosses the blood–brain barrier, and is readily converted into DA by the endogenous enzyme, aromatic L-amino acid decarboxylase (AADC). The long-term efficacy of levodopa, however, is limited by progressive degeneration of nigral cells, the source of AADC within the nigro-striatal system. Over time, increasing doses of levodopa are required for maintenance of clinical response, but dose escalations are ultimately limited by the development of dyskinesias as well as psychotic and autonomic symptoms. Moreover, with prolonged levodopa therapy, patients may also experience wearing off and "ON-OFF" phenomena.

Gene therapy for PD has focused so far on the significant motor deficits in the disease. The first human trial of gene transfer for PD involved delivery of GAD gene in an adeno-associated viral vector (AAV) to the subthalamic nucleus (STN) described in detail in the following chapter. Subsequent studies were directed at delivery to putamen of AAV serotype 2 (AAV2) encoding either AADC or the neurotrophic factors neurturin (NTN) or glial cell line-derived neurotrophic factor (GDNF). In addition, a lentivirus-based approach aimed at restoring DA synthesis to the putamen by delivery of the three components required for DA synthesis from tyrosine (tyrosine hydroxylase (TH), GTP cyclohydrolase, and AADC) has advanced into clinical study [4]. In this chapter, we address the challenges that had to be addressed in nonclinical and clinical studies to ensure effective distribution of vector. This factor, more than any other, explains much of the clinical data accumulated so far. Over the past decade, we have learned much more about how AAV vectors are transported by axons to sites distal from the site of injection. We have also learned more about the immunological consequences and caveats of AAV gene

therapy in the brain. These issues will be discussed and related to ongoing and future endeavors as we collectively fashion new gene-based medicines from decades of research.

AAV Delivery to the Putamen

A key problem in targeting the putamen with gene therapy is that it is a relatively large structure with an elongated anatomy and this tends to limit distribution of the therapeutic agent if simple parenchymal injections are used (Fig. 4.1). The putamen of an NHP is about sixfold smaller than that of a human adult [5]. These anatomical considerations have presented recurrent problems when trying to reproduce preclinical results in clinical trials [6–8]. To date, convection-enhanced delivery (CED) seems to be a more efficient system in order to achieve complete coverage of target structures. CED, first described by Oldfield and colleagues [9], is a parenchymal

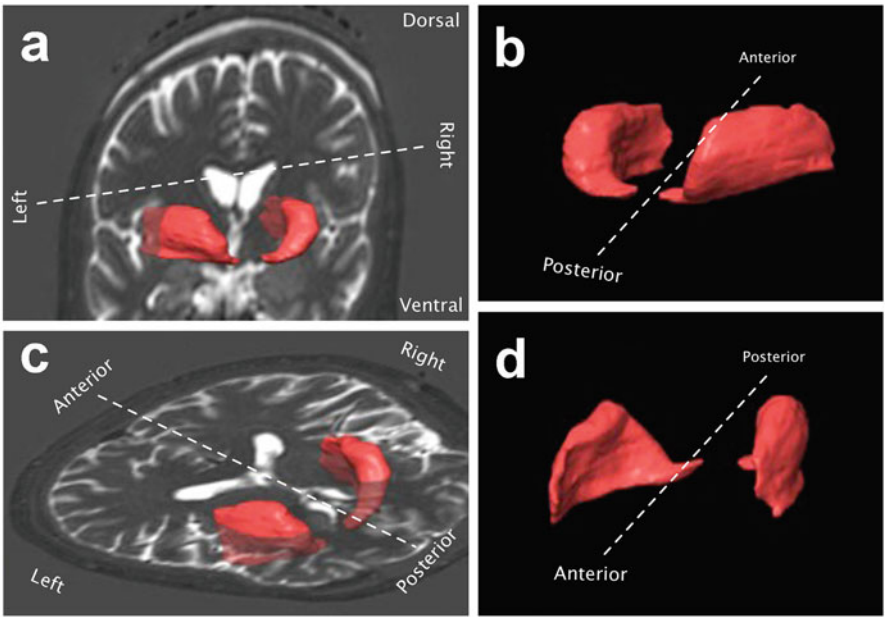

Fig. 4.1 3-D reconstruction of the human parkinsonian putamen based on T2 MRI. (**a**) Coronal view, (**b**) axial view, (**c**) posterior view, (**d**) anterior view. Volume of unilateral parkinsonian putamen is approximately 4000 mm^3. Delivery of viral vectors by direct putaminal infusion is challenging due to somewhat conical shape of the structure along the long axis of the nucleus. This means that trajectories must be carefully chosen to permit cannula placement such that each convection-enhanced delivery-based infusion fills as much of the tissue as possible without leakage into surrounding tissues or ventricles. In our estimation, at least a third of the targeted putaminal volume must be transduced in order to achieve therapeutic levels of expression

infusion technique that, by means of a pressure gradient from a cannula tip positioned within the target structure, generates bulk flow of macromolecules within the interstitial fluid (IF) space leading to displacement of IF by the infusate. This method requires the pressure at the tip of the cannula to exceed the interstitial pressure of the tissue, thereby allowing greater quantities of therapeutic agents to be distributed through large volumes of brain tissue from a single cannula via a pressure-driven engagement of the perivasculature to propel infusate over significant distances [10]. Our group has worked extensively in optimizing this method over the years in NHP [11–15]. Our current technique permits monitoring of parenchymal infusions by the inclusion of free gadoteridol, a tracer visible with magnetic resonance (MR), in the therapeutic agent preparation and performing them in an MR scanner. This method greatly enhanced the accuracy and effectiveness of AAV delivery since it provides real-time visualization of the infusion [11]. In fact, magnetic resonance (MR) tracers for real-time CED have been already used in 11 PD patients treated at NIH and UCSF, have been shown to be safe, and have proven highly informative regarding AAV delivery in the putamen (ongoing clinical trials). In our experience, and regardless of the encoded transgene, MR imaging (MRI) obtained while co-infusing AAV2 with gadoteridol shows an excellent correlation with the transgene expression as assessed by immunohistochemistry in NHP brain [13–16].

Because infusions can now be visualized, we have been able to define quantitative relationships between infusate volume (V_i) and subsequent volume of distribution (V_d) for both white and gray matter [17]. The use of real-time convective delivery (RCD) is quickly becoming central to neurological gene therapy because it allows the neurosurgeon to directly monitor the distribution of therapeutics within the brain and is currently being used in gene therapy for PD (see below) as well as in experimental treatment of glioblastoma with recombinant retrovirus [18] and liposomal Irinotecan [19]. Reflux along the CED cannula or leakage outside the target area, especially at higher flow rates, can be monitored and corrective steps taken, such as retargeting the cannula or altering the rate of infusion [20, 21].

During RCD, the V_d for a given agent depends on the structural properties of the tissue being infused, for example, hydraulic conductivity, vascular volume fraction, and extracellular fluid fraction [22]. It also depends on the technical parameters of the infusion procedure such as cannula design and placement, infusion volume, and rate of infusion [23–25]. The overall aim is to enhance infusate distribution within the target tissue but to limit the spread of the therapeutic agent into regions outside the target. Optimization of cannulae in pursuit of these objectives has been a critical, yet neglected, feature of brain delivery protocols. In early studies, we confirmed that smaller cannula diameters allowed faster delivery rates, but the smallest available cannulae were associated with increasing reflux when the rate of infusion exceeded 0.5 μL/min [26], clearly a significant problem when infusing large volumes. The key problem was reflux, defined as flow of infusate back up the outside of the cannula, leading to a loss of sufficient infusion pressure to force infusate out into the parenchyma. We examined several types of cannulae and concluded that a stepped design (Fig. 4.2) with a fused silica tip provided us with the most consistently

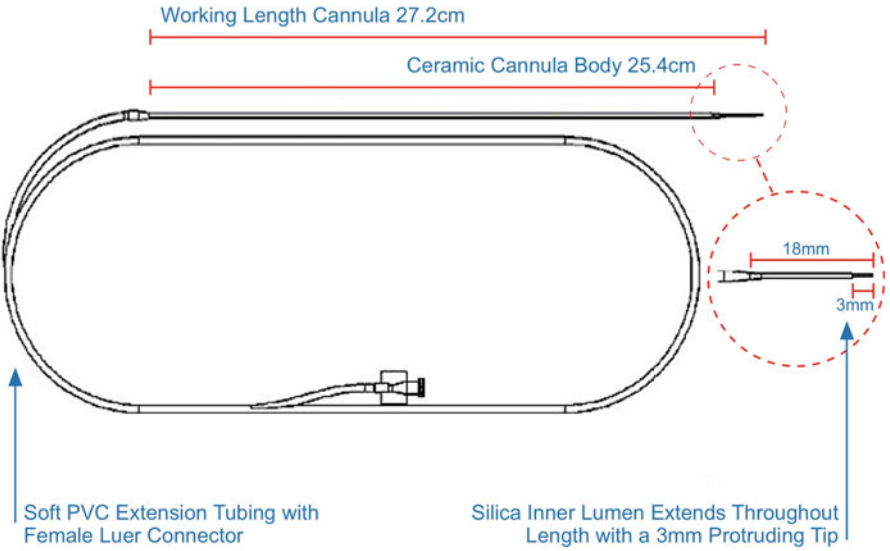

Fig. 4.2 Reflux-resistant infusion cannula. The cannula is made of MRI-compatible materials. It has a stepped distal tip with a 30-cm rigid ceramic shaft protecting the fluid-filled lumen. Soft tubing protects the lumen in the center portion and the distal end where it terminates at a female Luer connector. The lumen, also manufactured from nonreactive silica, extends beyond the end of the needle by up to 3 mm. The stepped design (silica step and ceramic step) enables the reflux resistance of this device

robust, reflux-free brain delivery [26–28]. We now see absence of reflux with infusion rates up to 30 μL/min, which greatly reduces infusion time compared to initial gene therapy clinical trials that used CED to deliver AAV2 [29–31]. The diameter of the larger stem of the cannula has an outer and inner diameter of 0.53 and 0.45 mm, respectively. The outer and inner diameters of the tip segment are 0.43 and 0.32 mm, respectively. Each infusion cannula included a distal tip that extended 3 mm beyond the end of the guide stem.

The distance from the cannula step to its entry point in the target region is a critical parameter. We defined optimal zones for cannula placement during RCD [5, 32] on the basis of containment of infusate within the target region. We defined a subset of cannula locations associated with complete, substantial, or poor containment within the target. Leakage of infusate into nearby ventricles or white matter tracts depended on the distance of the cannula tip from these structures. Such optimal placement is helping clinically to limit untoward distribution into white matter tracts [20].

To make RCD technology more clinically tractable, the ClearPoint® system (MRI Interventions Inc, Irvine, CA) has been adopted by us to translate targeting from the NHP brain into humans. ClearPoint is a novel integrated hardware (skull-mounted SmartFrame® device)/software platform for RCD that provides prospective stereotactic guidance for cannula placement [33] and performance of RCD (Fig. 4.3), and is already used in clinical studies to perform brain biopsies [34, 35]

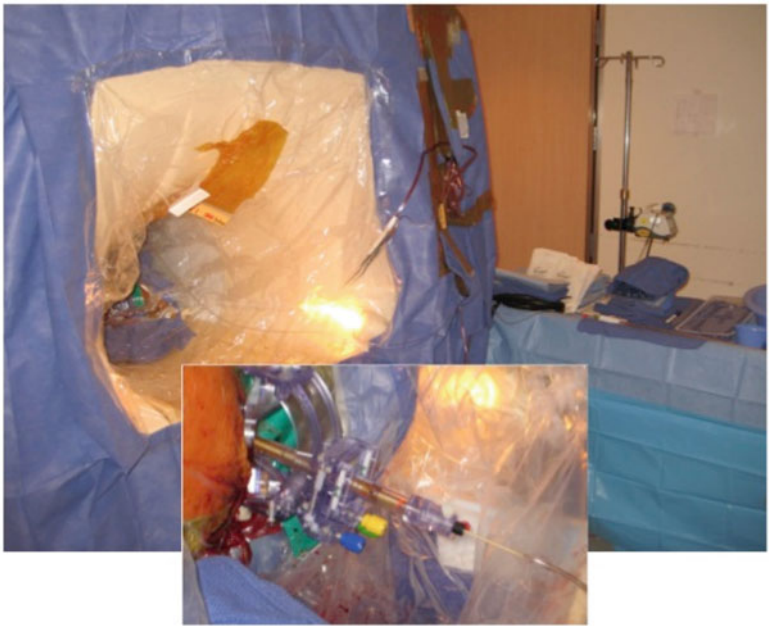

Fig. 4.3 ClearPoint® system for surgical procedure. The image shows the MR operating room set up for AAV delivery. A sterile field is established around the subject's head inside the MR scanner. Surgery is performed to mount the ClearPoint system onto the skull. *Insert*: Detail of the skull-mounted device and cannula already inserted and ready for AAV infusion into the target structure

and placement of deep brain stimulation (DBS) leads [36–38]. We are now using ClearPoint for RCD of AAV2 into the putamen of PD patients with a reflux-resistant MR-compatible infusion cannula (SmartFlow®). The targeting accuracy of this delivery system and the performance of the infusion cannula were validated in NHP [14]. Based on the results so far in placing DBS leads and AAV2 infusion in PD trials, ClearPoint appears to be highly accurate. Satisfactory cannula placement is routinely achieved without the need for repositioning [14, 15]. Targeting error, defined as the three-dimensional distance between the expected cannula tip location and the actual location, is about 0.8 mm in NHP studies [14], and sub-millimeter accuracy has been documented in human studies where ClearPoint has been used for placement of DBS electrodes [36–38]. ClearPoint has also allowed us to place two infusion cannulae close together (5-mm separation). In addition, we have found no difficulties in relocating a cannula from one site to another in a single infusion session. We have not experienced any cannula occlusion, reflux, or leakage into adjacent structures, and we have seen no evidence of persistent tissue damage. Similarly, we have been able to use MRI-based trajectory planning to avoid intra-operative hemorrhages in contrast to earlier trials (see below) where this system was not used. This new platform technology is now bearing fruit in current AAV2-based clinical trials.

Axonal Transport

AAV serotypes evince diverse tropisms in the central nervous system with attendant advantages and disadvantages. Commonly used serotypes of AAV are numbered from 1 to 12, but, as Wilson and colleagues have shown, infections occurring in nature probably generate from hundreds of variants [39], only some of which are infectious and even fewer retained in mammalian genomes. Only a handful of AAV vectors to date, however, have been examined in rodent and primate brain. A survey of PubMed citations indicated the dominance of AAV2 in the literature, followed by serotypes 1, 5, 8, and 9. This is not to say, of course, that other novel serotypes will not be advanced in the future, especially via intentional selection and engineering of capsids [40–45]. The prospect of this kind of engineering emphasizes the need for a highly standardized and reproducible vector delivery system in order to be able to assess the unique properties of newer AAV serotypes.

We have favored the use of neuron-specific serotypes primarily for safety reasons, since this restriction avoids targeting of antigen-presenting cells, addressed in more detail below. AAV2, the most widely used serotype clinically, is neuron specific in the brain and we recognized its propensity for anterograde axonal transport some years ago [46, 47]. Thus, AAV2 is transported from the soma to terminals projecting to distal structures and this transport results in release of intact AAV particles from axon terminals to transfect other nearby cells in the distal structure. For example, striatal infusion of AAV2 directed strong fiber transduction in the globus pallidus, SN pars reticulata, and STN, thereby demonstrating an anterograde striato-pallidal transport in NHP brain [46]. Similarly, thalamic infusion of AAV2 resulted, via robust anterograde thalamo-cortical transport, in strong transgene expression in motor and sensory cortical regions in rats [46, 48] and NHP [47]. In contrast, AAV serotype 6 (AAV6) is transported in a retrograde direction in rodent brain. Recently, we showed that striatal infusion of AAV6 resulted in extensive transduction of cortical and SN pars compacta neurons; both structures innervate the striatum [49]. In a recent experiment in NHP, we also found that putaminal AAV6-GFP undergoes similar retrograde transport when injected into putamen [50]. However, we occasionally observed transduced astrocytes, in contrast to experiments in rats [49]. The transduction of astrocytes by AAV6 appears to be relatively rare. We do not yet know, therefore, whether this has serious immunological implications, although we did observe MHC-II upregulation in transduced NHP astrocytes.

This remarkable directional divergence in axonal transport properties, apart from being intriguing at the molecular level, has clear therapeutic implications. By means of either AAV2 or AAV6, it is possible to target different distal structures even when the same anatomic region is the primary infusion site. Thus, the ability of AAV2 to be transported anterogradely supports its use in the treatment of PD motor deficits [31, 51] where transport of the vector throughout basal ganglia is largely confined to projections that do not degenerate in PD results in transduction of many structures that are affected in PD (Fig. 4.4) [46, 47].

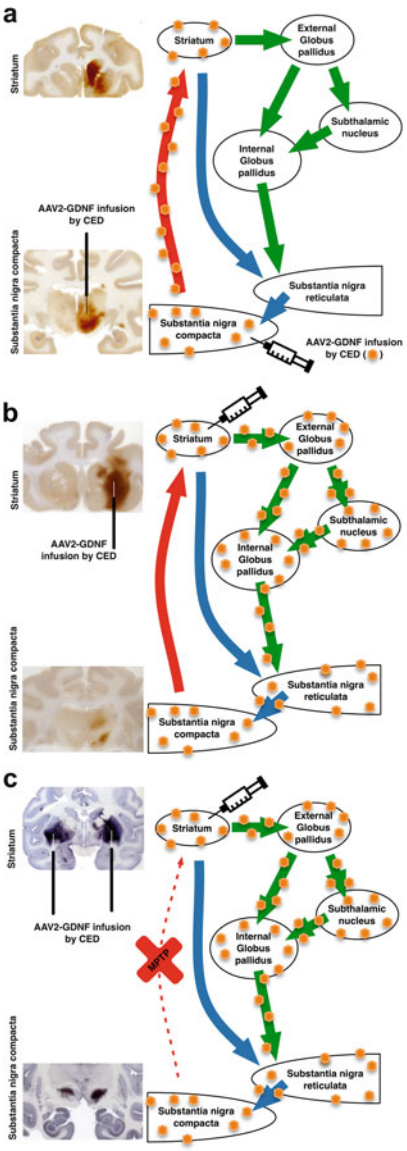

Fig. 4.4 AAV2-GDNF transport after infusion into the brain of naïve and parkinsonian nonhuman primates (NHP). The diagram shows the neuronal projection circuit between the different structures comprising the basal ganglia. (**a**) When infused by convection-enhanced delivery (CED) into substantia nigra (SN) of naïve NHP, AAV2-GDNF particles transduce dopaminergic nigral neurons and are subsequently transported to the striatum through the nigro-striatal axonal projections (viral particles along *red arrow*). (**b**) When delivered into the striatum (putamen), AAV2-GDNF particles enter the striatal medium spiny neurons and travel through the efferent basal ganglia structures (globus pallidi, subthalamic nucleus, SN pars reticulata) and reach the SN pars compacta by the projections sent from SN pars reticulata (viral particles along *green arrows*). However, nigro-striatal fibers in parkinsonian brains (**c**), both in patients and MPTP-treated NHP, are dramatically diminished and this prevents retrograde transport of any GDNF protein (*dotted red arrow*). On the other hand, efferent projections from the striatum and other basal ganglia nuclei are spared and, as shown previously in naïve NHP, can transport AAV2-GDNF vector to SN if delivered to the putamen (viral particles along *green arrows*)

The difficulty of achieving the requisite level of transgene expression over sufficient areas of the striatum to exert a positive effect in PD patients was encountered in a 58-patient-controlled (sham surgery) Phase 2 trial of AAV2-NTN (CERE-120). This trial was sufficiently powered to overcome the anticipated placebo effect prevalent in PD studies. In this study, a total of 16 vector injections (40 μL total per putamen) were made via 8 needle passes that resulted in only about 7.5 % coverage of the putamen, based on *postmortem* analysis of 2 participants in the study who had died from unrelated causes [6]. This result suggested that perhaps inadequate distribution of vector within the putamen was responsible for the lack of clinical effect. In a separate trial, where CED was employed, AAV2-hAADC distribution was greater as shown by positron emission tomography (PET) with the AADC-specific substrate, 6-[18F]-fluoro-metatyrosine (FMT) [7, 52]. In contrast to the above study, only two reflux-resistant infusion cannulae per hemisphere were employed in the AAV2-hAADC study. Distribution of the vector infusate in these patients was visualized by postoperative MRI within 7 h of vector delivery and was calculated to cover about 25 % of the post-commissural putamen on each side of the brain [30]. The CED approach required a smaller number of infusions and, therefore, presumably lowered the risk of adverse events associated with intracranial cannula placement. It is, however, evident from these early studies that, without a standardized delivery system that can be implemented in multicenter studies and reliably provide a larger distribution of vector within the target brain structure, it will not be possible to fully evaluate the efficacy of therapeutic gene therapy vectors. Even in the successful AAV-GAD study, where gene transfer was targeted to the relatively small STN, it was found that precise targeting was critical to clinical benefit and no motor improvements were observed when the cannula was not correctly positioned [53].

In fact, based on extensive NHP studies and current clinical AAV trials where we delivered AAV2 vector by RCD, it is clear that transduction efficacy is directly correlated more to the volume that is being delivered rather than the number of vector genomes (vg) alone as has been suggested by others [54]. Ideally, where the total V_i to the putamen distributes in the target regions without any reflux or leakage along perivascular tracts, the ratio of V_i to the V_d is 1:3. For example, infusion of 100 μL of AAV2 will fill 300 mm^3 of the putamen. Figure 4.5 illustrates the delivery volumes of viral vector in PD trials. The first AAV2-NTN trial delivered 40 μL per putamen, via simple needle injection at eight sites in the putamen, resulting in NTN expression confined only to the injection loci [8]. The first AAV2-hAADC trial, conducted at UCSF and Jichi Medical University, employed 100-μL infusions with CED at two sites with a reflux-resistant cannula [26]. This delivery could only cover 300 mm^3 of the putamen, consistent with our post-infusion T2 measurements [30]. In the second AAV2-NTN trial, investigators used 150 μL per putamen (three sites; 50 μL per site). In the best-case scenario, if a reflux-resistant cannula had been employed, the infusion would have resulted in about 450 mm^3 of putaminal coverage, representing around 12 % of the putamen. In the most recent group of patients in the ProSavin® trial, 300 μL of lentivirus vector is being infused with CED possibly resulting in 900 mm^3 of putaminal coverage, if indeed the lentivirus distributes in the same fashion as AAV2. In the ongoing MR-guided clinical trials of AAV2-

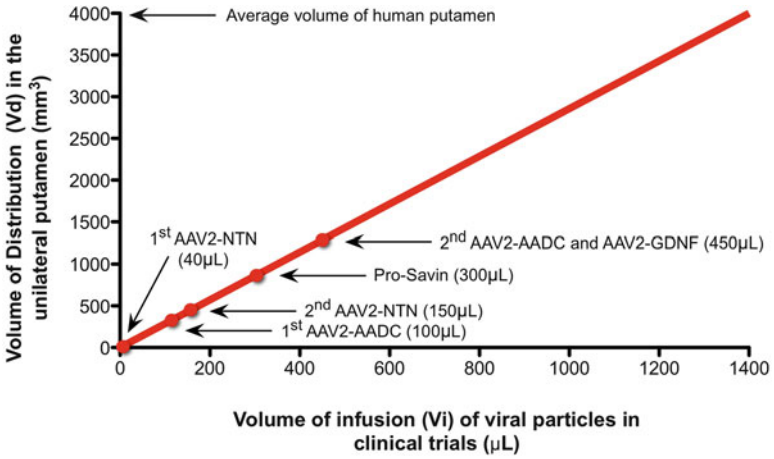

Fig. 4.5 Comparison of target structure coverage on different gene therapy clinical trials for Parkinson's disease. The plot of volume of vector distribution (V_d) vs. the volume of infusion (V_i) shows a positive linear correlation between the two variables. Increasing V_i (from 40 µL in the first trial to 450 µL in the latest) results in a parallel increase in V_d with a slope of 1:3. Although putaminal coverage has been significantly optimized since the first gene therapy trial (V_i of 450 µL covers approximately 1400 mm^3), preclinical and clinical data suggest that higher volumes could be delivered to achieve further coverage of the structure (mean volume is 4000 mm^3)

hAADC and AAV2-GDNF (UCSF and NIH), 450 µL of AAV2 is being infused in each putamen [15]. Intra-operative MRI of the gadoteridol contrast reagent included in the vector infusion confirmed (unpublished data) that V_d is approximately threefold V_i in the putamen, giving a V_d of about 1500 mm^3, predicting transgene expression in about 50 % of the putamen or 80 % of the post-commissural putamen [55]. Since AAV administration is monitored by co-distribution of MRI contrast reagent, V_d can be measured dynamically throughout the infusion, thereby permitting calculation V_i/V_d ratios over a range of V_i between 10 and 450 µL.

Our experience suggests that vector dose-ranging experiments should start with a consideration of the volume of the anatomical structure being targeted. That information predicts an optimal infusion volume and, within that constrained volume, various concentrations of vector can be tested. In practice, however, the shape of the target will tend to make this more of an aspiration than a precise reality. The primate putamen is a somewhat conical structure that necessitates innovation in cannula trajectory and cannula design, currently the focus of our research.

Clinical Experience with AAV2-hAADC for PD

Striatal neurons do not degenerate in PD and are capable of long-term expression of transgenes [56, 57]. In the AAV2-hAADC approach, it was hypothesized that by increasing striatal AADC levels sufficiently [58], PD patients would reestablish appropriate responsiveness to exogenously administered levodopa, thereby reducing

dose requirements and the long-term adverse effects associated with escalating levodopa therapy. The rate-limiting step of intrinsic dopamine synthesis involves an earlier enzymatic step, TH; thus, AADC activity is expected to be limited by exogenous levodopa dosage. This ability to regulate DA production and concentration by adjusting pro-drug (levodopa) doses offers a key safety component of this therapy.

The AAV2-hAADC vector has been under investigation for more than a decade and its viability as a potential treatment for PD is supported by numerous animal studies [56–59]. Importantly, it has been demonstrated that striatal administration of AAV2-hAADC to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-lesioned Rhesus monkeys (a commonly used nonhuman primate model of PD) resulted in statistically significant long-term (8 years) improvement in behavioral rating scores in parkinsonian NHP, significantly lowered levodopa requirements, and reduced levodopa-induced side effects [57]. Moreover, PET with FMT, a levodopa analogue for in vivo analysis of transgene expression, confirmed persistent AADC activity for the duration of the study, indicating that infusion of AAV2-hAADC vector into NHP brain results in at least 9 years of transgene expression [56, 57].

The available nonclinical and clinical data available to date suggest that AAV2-hAADC gene transfer therapy offers promise for PD patients experiencing a decline in levodopa efficacy and an increase in side effects associated with standard pharmacotherapy. A Genzyme-sponsored, Phase 1, open-label safety study of striatal administration of AAV2-hAADC was initiated in 2005 in patients with mid- to late-stage PD under Investigational New Drug (IND) application (IND #11366) [7, 31, 60]. Ten patients were treated and monitored for up to 5 years. A third, higher dose cohort was planned but not enrolled due to AAV2-hAADC manufacturing limitations. A second Phase 1 investigator-initiated study in six patients with PD was conducted at Jichi Medical University, Japan, with the same vector and delivery procedure [52]. Based on available data from the two Phase 1 studies, the AAV2-hAADC striatal approach was well tolerated.

In the UCSF study, a total of 10 subjects were enrolled, with 5 subjects assigned to each of two dose cohorts: nominally 9×10^{10} and 3×10^{11} vg per subject (later qPCR analysis of the vector indicated that the respective doses were actually 2.3×10^{11} and 7.5×10^{11} vg). The vector was administered in a total volume of 200 μL distributed over four sites: two sites each in the left and right putamen at 50 μL per site. Subjects were monitored every 2 weeks for the first 2 months after surgery, with continued monthly follow-up for the remaining year, and every 3 months for the next 4 years. The two dose cohorts were serially enrolled. Escalation to the higher dose was contingent on the demonstrated safety and tolerability of the previous dose. There was a period of more than 10 months between treatment of the last subject in the first dose cohort and treatment of the first subject in the second dose cohort [31]. In the investigator-initiated Phase 1, open-label, single-dose safety study at Jichi Medical University study in Japan, the same AAV2-hAADC product and delivery protocol was used as in the UCSF study [52]. Six PD patients were enrolled into a single-dose cohort (nominal 3×10^{11} vg per subject; actual dose after re-quantitation was 7.5×10^{11} vg). A total of 16 subjects received AAV2-hAADC in these two studies. Nine out of 10 subjects in the initial

Genzyme-UCSF study completed a 5-year follow-up with one subject only attending the first-year follow-up visits. Four out of 10 subjects subsequently underwent DBS at 10, 18, 30, and 50 months after AAV2-hAADC delivery. All subjects from this study are alive and ambulatory.

All 10 subjects in the initial Genzyme Phase 1 study experienced at least one treatment-emergent adverse event (TEAE). The majority of TEAE were mild in intensity. The most common TEAE were incision site pain (9/10 subjects [90 %]), fall (8/10 subjects [80 %]), and procedural headache (headache due to the procedure in 6/10 subjects [60 %]). All subjects (10/10; 100 %) experienced at least one TEAE that was considered by the investigator to be related to the *study procedure*. The following TEAE were considered by the investigator to be procedure related: incision site pain, procedural headache (headache due to the procedure), transient facial edema, subdural hematoma, hypoesthesia, intracranial hemorrhage, headache, cerebral infarction, encephalomalacia, gliosis, paresthesia, subarachnoid hemorrhage, face edema, pyrexia (also considered related to investigational product and to study device), pain of skin, peri-orbital edema, nausea, vomiting, eye edema, abnormal brain MRI (i.e., hyperintensity along the therapy tract of uncertain significance that appeared decreased on follow-up MRI; also considered related to investigational product and to study). TEAE related to the investigational product were experienced by 3/10 subjects (30 %). The following TEAE were considered related to the *investigational product*: fall in one subject; headache in one subject; and pyrexia and abnormal brain MRI (i.e., hyperintensity along the cannula insertion tract of uncertain significance that appeared decreased on follow-up MRI), both of which occurred in the same subject, and both of which were also considered related to the procedure and to the study device. TEAE related to the *study device* were experienced by 1/10 subjects. The only TEAE considered device related were the aforementioned events of pyrexia and abnormal brain MRI, both of which occurred in the same subject. It was reported that all subjects (6/6) in the Jichi Medical University Phase 1 study experienced mild, transient headache around the surgical burr-holes for 2 days after surgery.

Four of the 10 subjects, who enrolled in the initial Genzyme-UCSF Phase 1 study, experienced at least one serious adverse event (SAE). A total of eight SAEs were reported among these four subjects. The SAEs experienced by each of the four subjects are as follows:

- One patient from the low-dose cohort experienced an asymptomatic intracranial hemorrhage secondary to a cerebral venous infarction in the left frontal lobe on Day 0 and was probably related to the surgical procedure. Approximately 13 months after administration of the investigational product, this subject underwent hip replacement surgery (due to arthritis present prior to participation in the study), complicated by femur fracture. This SAE was not considered related to the procedure, study device, or investigational product.
- Subject 110 (high-dose cohort) experienced a symptomatic intracranial hemorrhage on Day 0, considered by the investigator as definitely related to the study procedure.

- Subject 111 (high-dose cohort) was hospitalized for elective transurethral resection of the prostate on Day 76 due to benign prostatic hypertrophy. This SAE was not considered related to the study procedure, study device, or investigational product.
- The two SAEs of intracranial hemorrhage (one subject in each dose cohort) and the cerebral venous infarction (subject in low-dose cohort) were early events that occurred on the day of surgery (temporally related) and were considered by the investigator to be related to the study procedure. The other five SAEs [Parkinson's disease (deep brain stimulation for refractory PD), urinary tract infection, hip surgery, femur fracture, and benign prostatic hyperplasia] were late events occurring several months after surgery, and were not considered related to the investigational product, study device, or study procedure.
- One SAE was reported from the six subjects enrolled in the Jichi Medical University Phase 1 study. Subject A-2 experienced a venous hemorrhage in the right frontal lobe beneath and anterior to the burr-hole, which resulted in symptoms related to hemorrhage and surrounding edema. This SAE was considered by the investigator to be related to the surgical procedure.

Gene expression in the striatum was assessed by PET scanning with FMT as a tracer. AADC enzyme activity was substantially increased over baseline for both dose cohorts during the observation period. Subjects in the Genzyme-sponsored Phase 1 study received FMT-PET scans at approximately 1, 6, 12, 24, 36, 48, and 60 months after surgery. FMT uptake initially increased by a mean 25 % and 65 % within the region of the putamen that was treated by AAV2-hAADC for the low- and high-dose cohorts, respectively, compared with baseline, and remained consistent throughout the entire study duration. A mean 56 % increase in FMT uptake was reported in the first two patients in the Jichi Medical University Phase 1 study (equal to high-dose cohort).

The Unified Parkinson's Disease Rating Scale (UPDRS) measures PD symptomatology and treatment side effects in four domains. Part I consists of four questions that assess mentation, behavior, and mood. Part II consists of 13 questions that assess activities of daily living. Part III consists of 14 questions related to motor function (e.g., tremor, bradykinesia, gait, and rigidity), and Part IV consists of 11 questions related to complications of therapy. For each domain, a higher score indicates greater problems. The total UPDRS score is defined as the sum of Parts I, II, and III, and it ranges from 0 to 199. The defined OFF state is when the subject has been off PD medications for 12 h, or for an established longest tolerable interval if less than 12 h. The defined ON state is when the PD medications have the greatest functional benefit, in the opinion of the subject and investigator. In general, the UPDRS Part II scores did not show improvement at the time points through Month 3 and the UPDRS Part III scores showed little to no improvement after treatment. At Month 6 and time points beyond Month 6, the mean total UPDRS scores and mean motor scores (UPDRS Part III) in both the ON and OFF states improved after treatment for both dose cohorts (i.e., mean scores decreased relative to baseline). A slow deterioration in UPDRS scores was apparent beyond 12 months.

Post-treatment improvements were also seen in subject diary measures at the 6-month time point. The mean number of waking hours spent in the OFF state for all subjects in the Genzyme Phase 1 study was reduced by 3.1 h after surgery, and mean ON time increased by 3.3 h. These improvements were not associated in an increase in severe dyskinesia. Similar improvements in patient mobility were reported from the Jichi Medical University study. 6 months after surgery the daily dose of DA mediation was reduced in 8 of 10 subjects (80 %) in the Genzyme study and 3 of 6 subjects (50 %) in the Jichi Medical University study. No subjects required an increase in dopaminergic mediation dose. This reduction in optimal dose is anticipated as increased AADC activity will enhance conversion of levodopa to dopamine. Transient increases in dyskinesia were reported but resolved with the reduction in dopaminergic mediation dose. In summary, preliminary analysis of efficacy data from the previous uncontrolled Phase 1 studies suggests that administration of AAV2-hAADC increased AADC enzyme activity in the striatum (as measured by FMT-PET), improved UPDRS scores (both in the OFF and ON states), decreased the percent of waking hours spent in the OFF state, and increased the percent of waking hours spent in the ON state without dyskinesia.

A new UCSF investigator-initiated Phase 1 study incorporates recent advances in intra-operative MRI-monitored CED that enhances targeting accuracy and provides greater control over direct drug delivery to the brain [14]. This study extends the prior investigations of striatal AAV2-hAADC delivery to PD patients by using larger infusion volumes with real-time MRI visualization (to improve vector distribution and accurate cannula placement) and a higher dose of AAV2-hAADC to test for improved clinical effect. Patients will be monitored for 3 years. This ongoing UCSF study is enrolling at the time of writing 10 PD patients with fluctuating responses to levodopa. The initial five subjects will receive a moderate dose of AAV2-hAADC (7.5×10^{11} vg per subject) and the subsequent five subjects a higher dose (2.3×10^{12} vg per subject). The vector will be administered in a total of 900 μL spread over four sites: two sites in each the left and right putamen. The larger delivery volume relative to the previous studies is intended to increase distribution of gene transfer within the putamen and accommodates delivery of the higher dose without contending with manufacturing limitations. The real-time MRI monitored delivery provides additional safety by minimizing the risk of off-target delivery. Subjects will be monitored regularly with 10 study visits during the first 12 months and semiannual clinic visits for the following 2 years.

Neurotrophin Gene Therapy

Although medications can temporarily alleviate the symptoms of PD, they do not influence the degenerative process. Progressive loss of nigral dopaminergic (DA) innervation (the pathological hallmark of PD) results in progressive catecholaminergic dysfunction and death. GDNF was first identified based on its ability to promote the survival of embryonic DA neurons in vitro, and research has demonstrated

beneficial effects of GDNF in animal models of PD. Preliminary clinical trials of recombinant GDNF infusions yielded inconclusive results. Observed problems with tolerability and efficacy in these studies may have been related to the methods of delivery. Recent evidence indicates that gene transfer via direct delivery of viral vectors may represent a superior approach for the treatment of PD with GDNF.

GDNF was isolated from the B49 cell line based on its ability to promote the survival of embryonic DA neurons in vitro [61–63]. GDNF was the first identified member of a group of neurotrophic factors related to the basic fibroblast growth factor family. NTN, persephin, and artemin were subsequently identified [64]. A gene transfer clinical trial with AAV-NTN was recently undertaken at UCSF [8, 51]. GDNF and its family members act through a novel receptor signaling system composed of a GPI-linked neurotrophic factor binding subunit and this complex in turn activates the C-ret tyrosine kinase transmembrane receptor, reviewed in reference [64]. A multitude of preclinical studies with GDNF protein in an array of rat, mouse, and monkey models of PD have demonstrated potent effects of this factor in protecting DA neurons from neurotoxin-induced cell death and in ameliorating indices of DA-dependent behaviors, reviewed in reference [65].

Delivery of the GDNF gene via recombinant viral vectors to mouse, rat, and NHP models of PD has been extensively investigated by many independent research groups with significant protection of DA neurons and attenuation of DA-dependent behavior deficits consistently reported, reviewed in reference [66]. Initial proof-of-concept studies were performed in Dr. Bohn's laboratory with an adenoviral vector to deliver GDNF gene prior to 6-OHDA lesioning in rats [67, 68], and subsequently confirmed by another laboratory with an AAV2 vector [69]. These early rodent studies independently demonstrated that delivery to the SN enhances protection of DA neurons against 6-OHDA-induced toxicity, but that GDNF delivery to the striatum is necessary to maintain the DA terminals and their synaptic function [70]. A more recent investigation by Eslamboli and colleagues demonstrated that very low levels of continuous GDNF expression (three-fold above baseline) after AAV gene transfer to the NHP striatum are sufficient to protect DA neurons and attenuate behavior deficits [71].

In recently performed experiments, our laboratory has investigated AAV2-GDNF delivery to the putamen of NHP (Rhesus macaques). Studies were undertaken to primarily assess the safety and feasibility of delivering very high doses of AAV2-GDNF to both the aged normal and a DA-depleted NHP brain as representative models of the PD brain [72–74]. This is in fact the only published study in which AAV2-GDNF (or AAV2-NTN) was evaluated in MPTP-lesioned animals after stable PD signs had been established for more than 6 months. This experimental paradigm more closely mimicked clinical reality and demonstrated the neuro-regenerative potential of growth factor administration. Other studies administered growth factors before, or shortly (days) after, toxic insult, thereby demonstrating protective rather than restorative properties of growth factors. This is a very important difference, since it is likely that the mechanism of growth factors required for protection vs. regeneration of DA neurons in the human brain is different and likely depends on dose and distribution within the brain (Fig. 4.6). For example, administration of growth factors

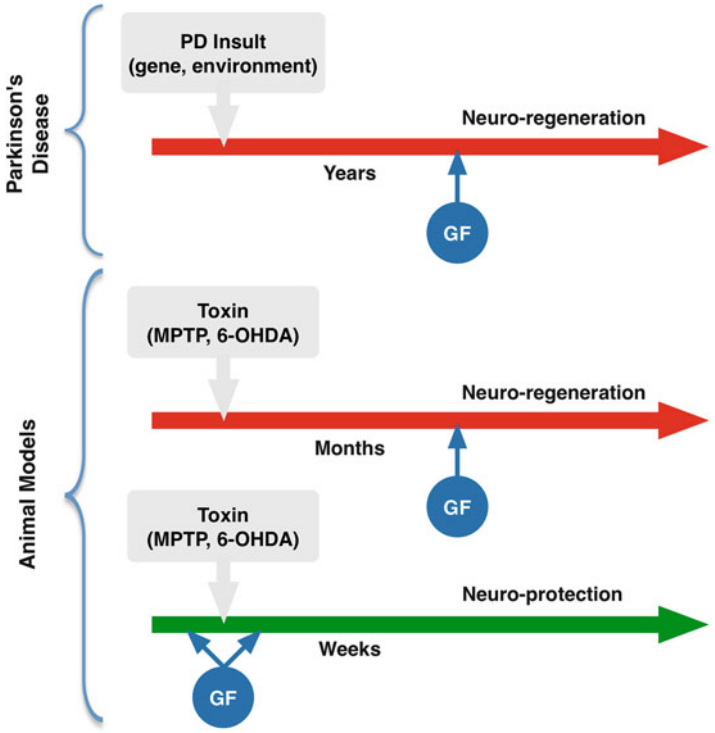

Fig. 4.6 Mechanisms underlying growth factor efficacy in Parkinson's disease and animal models of Parkinson's disease. (**a**) Delivery of growth factors into the putamen of PD patients takes place years after the onset of PD. At that point the progression of the disease has affected the nigro-striatal pathway. Intended treatment with growth factors is aimed at neuro-regeneration of DA innervation. Therefore, (**b**) therapeutic properties of growth factors should be tested in parkinsonian animal models months after inducing the dopaminergic lesion (administration of 6-hydroxydopamine (6-OHDA) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)). AAV2-GDNF administration in monkeys that were lesioned with MPTP 6 months earlier resulted in significant recovery from parkinsonism by means of neuro-regenerative mechanisms such as dopaminergic axonal sprouting and upregulation of TH in the nigral neurons. These results led to the ongoing clinical trial of AAV2-GDNF in PD patients. In contrast, (**c**) when treating animals before or immediately after (days or weeks) initiating lesion of the dopaminergic system, the neuro-protective properties of growth factors predominate. Since growth factors are delivered at the time of, or shortly after, the toxic insult, the nigro-striatal pathway is not fully damaged and these fibers can transport growth factors expressed in the putamen back to nigral neurons, thereby leading to neuro-protection

in the putamen at a time when nigro-striatal projections are still intact would permit growth factors to be transported in a retrograde fashion back to the dopaminergic nigral neurons, leading to at least partial protection of the nigro-striatal innervation. This mechanism is not in place when nigro-striatal degeneration has already occurred, a process that takes at least 4–6 weeks in NHP [75]. Administration of growth factors via gene transfer technology will have to be more complete and would need to involve

anterograde axonal transport via intact striato-nigral projection in the absence of a functional nigro-striatal pathway (refer to previous section in this chapter that deals with it in more detail, also Fig. 4.4). In addition, clinical trials in which growth factors are being investigated are not designed to test protection or slowing of PD progression, but clinical recovery, which can only be modeled in NHP models where nigro-striatal projections have degenerated commensurate with the kind of damage seen in at least moderately progressed PD.

Two doses of AAV2-GDNF were infused into the putamen of aged NHP (1.65×10^{11} and 1.65×10^{12} vg) both of which resulted in measurable enhancement of nigro-striatal function without raising any safety concerns: no downregulation of the DA system, no neuronal degeneration in the SN, no microglia activation, and no significant neuropathology findings. PET imaging showed an increase in AADC activity (FMT) in the striatum ipsilateral to the AAV2-GDNF infusion (Fig. 4.7) that was paralleled by an enhancement of locomotor activity, suggesting improved dopaminergic activity in the aged brain. *Postmortem* quantification of DA and metabolites (HVA and DOPAC) found increased DA turnover in the ipsilateral caudate-putamen [72] that was consistent with an increase in TH fiber density in the putamen and suggestive of GDNF-induced sprouting of the DA terminals. DA cell counting in the SN revealed the absence of either neurodegeneration or neurogenesis, although bilateral upregulation of TH staining in the nigra and medial forebrain bundle is indicative of increased cellular function after AAV2-GDNF infusion (Fig. 4.8). Neuropathological analysis for activated microglia and astrocytes found no safety concerns due to AAV2-GDNF treatment.

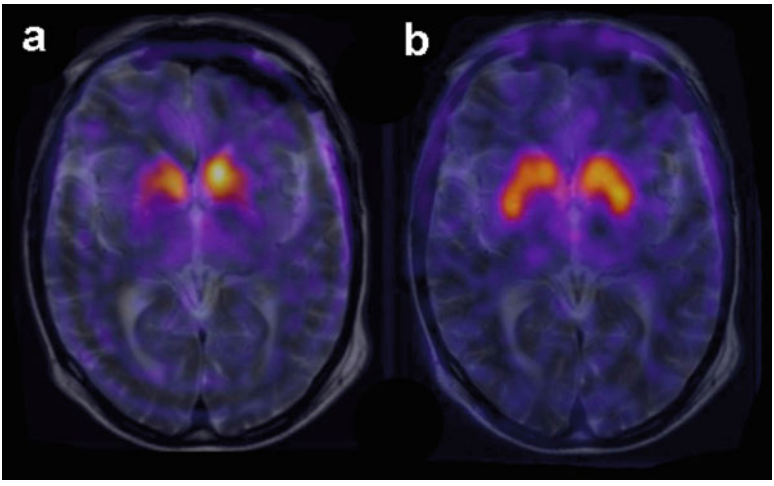

Fig. 4.7 Dopaminergic function restoration in the putamen after bilateral delivery of AAV2-hAADC. Superimposition of MRI image and FMT-PET scan of a parkinsonian patient before (**a**) and 3 months after (**b**) receiving bilateral CED of AAV2-hAADC. Note the bilateral recovery of AADC activity in the putamen after AAV2-hAADC infusion compared to the low FMT signal (*yellow*) before the infusion

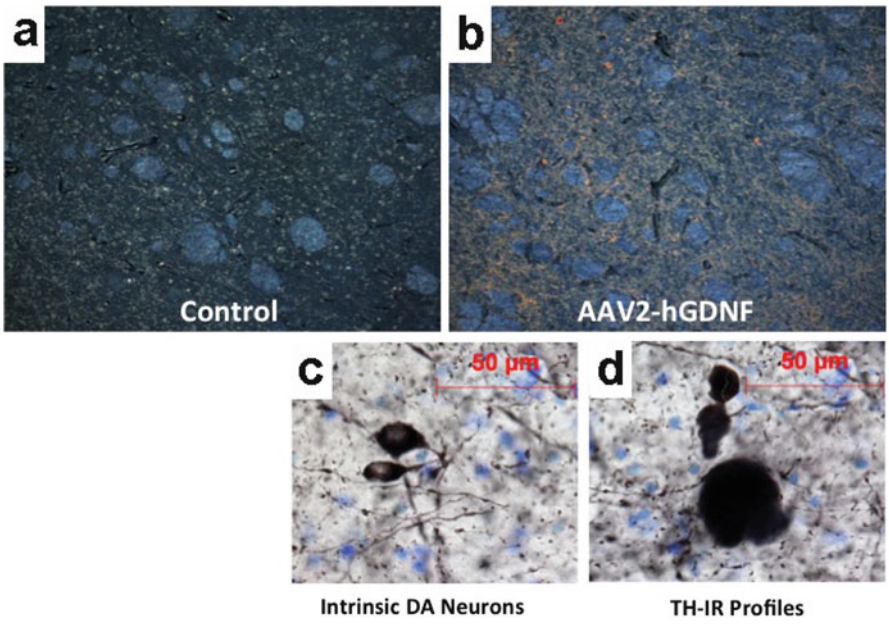

Fig. 4.8 Dopaminergic sprouting after striatal delivery of AAV2-GDNF in parkinsonian nonhuman primates. Panels show tyrosine hydroxylase (TH) staining in dark-field (**a**, **b**) or light-field microscopy (**c**, **d**). Compared to control striatum (**a**), striatal infusion of AAV2-GDNF (**b**) triggers dopaminergic sprouting that results in a dramatic increase of TH-immunoreactive (IR) fibers (*yellow*) in the striatum. Similarly, GDNF increases the number of intrinsic dopaminergic neurons in the striatum (**c**) and the appearance of TH-IR profiles (**d**) in the dopaminergic fibers that correspond to axonal varicosities

In the second NHP study in the Bankiewicz laboratory, delivery of AAV2-GDNF (5×10^{11} vg) to the putamen of MPTP-treated primates with stable PD signs for at least 6 months was shown to significantly attenuate parkinsonian-like behavior symptoms assessed by the modified PD clinical rating scale (CRS) [74]. Responsiveness to L-dopa administration was maintained at a similar level for both AAV2-GDNF and control monkeys with no hyperactivity observed in any of the animals. Improvements in CRS scores at 6 months were directly correlated to increases in PET-FMT uptake measurements relative to baseline levels. Similar to the neurochemical changes found in the aged primate study, DA and metabolites were consistently increased in the parkinsonian animals that were treated with AAV2-GDNF compared to PBS-treated controls. High DA turnover, 1 month after AAV2-GDNF infusion, was modestly reduced in animals 6 months after treatment, and these animals had higher DA levels than 1-month animals, suggesting an enhancement of DA function in AAV2-GDNF-treated animals. Histological analysis of brains collected 1, 6, 12, and 24 months after surgery did not trigger any safety concerns. Overall, the accumulation of encouraging safety data and efficacy from multiple independent GDNF gene delivery studies in animal models of PD indicate

that the experimental treatment proposed in this clinical trial holds great promise for producing positive neurobiological and clinical effects in patients with PD.

Clinical investigation of NTN gene therapy preceded initiation of our AAV2-GDNF Phase 1 study currently under way at NIH Clinical Center in Bethesda, MD (ClinicalTrials.gov Identifier: NCT01621581). The AAV2-NTN Phase 1 study involved AAV2-mediated bilateral stereotactic delivery to the putamen of the cDNA encoding human NTN. Delivery of AAV2-NTN was accomplished by a series of small injections throughout the putamen [8, 51], whereas the CED method in the ongoing AAV2-GDNF trial requires only two infusion sites. The initial safety and tolerability study, sponsored by Ceregene Inc., involved eight patients at UCSF and four at Rush University Medical Center [51]. Twelve patients were treated in two dose cohorts, low [1.4×10^{11} vg, $N=6$] and high (5.7×10^{11} vg, $N=6$). No serious adverse events were reported. Outcome data for the first eight patients showed an improvement in the 6-month postoperative UPDRS-III off scores of 27.9 % (±14) in the low-dose cohort and 51.2 % (±19.9) in the high-dose cohort. In the first 6 months, patients showed a greater than 50 % reduction in OFF time and a doubling in ON time without dyskinesias. At 12 months, both dose cohorts showed a greater than 35 % improvement in UPDRS-III scores when OFF. A multicenter, randomized, double-blind trial (including sites at UCSF and Rush) with patients randomized in a 2:1 ratio to receive either active therapy at 5.7×10^{11} vg ($N=34$) or sham surgery ($N=17$) was completed in October, 2007. In a November, 2008 press release (www.ceregene.com/press_112608.asp), Ceregene reported no appreciable difference between patients treated with AAV2-NTN versus those in the control group [8].

Investigators proposed changes in delivery method in a second Phase 2 trial that preceded the first negative trial [76]. Unfortunately, this trial also resulted in negative outcome. Strong placebo effects and the advanced stage of the study subjects were suggested as causal in the failure of the trial. However, suboptimal vector delivery in this second Phase 2 trial is likely to have played a major role as well. As we argue above (Fig. 4.5), administration of 150 μL of AAV2-NTN in best-case scenario would likely only cover 12 % of the putamen. In addition, NTN, unlike GDNF, does not appear to be secreted from the transduced cells in vivo, which limits its potential for any diffusion within the putamen. We believe that clinical efficacy may require significant transduction of medium spiny neurons in the parkinsonian putamen to overcome the profound GDNF resistance apparent in the disease as we have recently shown [77].

Conclusion

The advent of MRI-guided infusions, advanced planning software, and new types of cannulae promises to bring to the clinic a new, highly effective therapeutic technology in which viral vectors like AAV2 will be used routinely in neurosurgical suites to restore function in degenerated tissues. The speed and accuracy of even present technology is likely to make neurological gene therapy commonplace in the next

decade. The delivery platform we have helped develop is also finding application in the delivery of recombinant proteins and nanoparticles in both PD and brain malignancies. It is a major goal of our group to assist in the widest possible adoption of MRI-guided convective delivery technology.

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Chapter 5
GAD Gene Therapy for Parkinson's Disease

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Abstract The ability to directly modulate intracellular processes through genetic manipulation has long been felt to have great potential for treating intractable neurological and psychiatric diseases. To date, the largest number of clinical trials of central nervous system gene therapy has been in patients with Parkinson's disease (PD). Most of these have used adeno-associated virus (AAV) as a vehicle, which we demonstrated to be safe and effective for stable gene therapy in the brain more than 20 years ago. Here, we describe the development and results of the first human gene therapy for PD, which used AAV to transfer the gene for glutamic acid decarboxylase (GAD) into the subthalamic nucleus (STN). The STN is in the basal ganglia circuitry which is dysfunctional in PD, and human therapy for drug resistant PD has focused upon either lesioning or electrical stimulation of the STN for many years. In an initial open label pilot study, unilateral injection of AAV-STN into the STN of the more symptomatic hemisphere demonstrated safety and suggested evidence of efficacy based upon both motor improvements and reversal of functional imaging abnormalities up to 1 year. This led to a randomized, double-blind phase II clinical trial of bilateral AAV-GAD into the STN compared with patients receiving bilateral sham surgery. This confirmed the effectiveness of AAV-GAD, as the treated patients showed significantly greater improvements than the sham patients throughout both the 6-month blinded phase and full 12-month study phase, again with a very good safety profile. Functional imaging further supported these findings and identified a pattern of changes unique to the sham patients with improvement which was not seen in either the AAV-GAD patients or sham non-responders. These combined data support ongoing development of AAV-GAD as the only gene therapy in the CNS to date to demonstrate efficacy compared with contemporaneous sham controls and provide a stronger foundation for the further development of CNS gene therapy for a variety of disorders.

Keywords Parkinson's disease • Adeno-associated virus • AAV • Glutamic acid decarboxylase • GAD • Sub thalamic nucleus • STN • Positron emission topography • PET • Gene therapy

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Background

Gene therapy has long held promise as a cutting edge approach to exploit the power of gene technology for improving human disease. This has been particularly true in the nervous system, where major advances for devastating degenerative disease have been limited. One disease which has been a focus of novel biological approaches, such as gene therapy and cell transplantation, has been Parkinson's disease. This devastating disorder is characterized by loss of dopaminergic neurons of the substantia nigra, as well as other neuronal populations, leading to a characteristic movement disorder including resting tremor, muscular rigidity, and difficulty initiating movements. Medical therapies are very effective early in the disease, but reduced benefit and medication-related complications often lead many patients to seek alternative treatments over time [1]. Surgical therapies, in particular deep brain stimulation, can provide great benefit to patients, but require implants of electrical devices in the brain and body which also require frequent adjustments initially and some maintenance over time, and can lead to hardware-related complications [2]. Parkinson's disease can also be modeled in both rodents and primates, traditionally through chemical destruction of nigral dopamine neurons. Although the predictive value of these models for therapeutic success in humans has been questioned, the availability of these rodent and primate models provides opportunities for testing novel therapies in systems which more closely mimic human disease than many other neurological disorders. This combination of unmet need, and models which reflect at least in part the pathophysiology of the human disorder, has made Parkinson's disease a major focus of novel biological therapies such as gene therapy.

Translation of central nervous system gene therapy into a clinical reality was first facilitated by the identification of transfer agents which would be safe and effective in the human brain. It was recognized early that modified viruses could be very efficient vehicles for delivery of genes into mammalian cells. These initial viral vectors were largely based upon viruses which naturally infected the target organ [3–5]. This included herpes simplex virus vectors, which we and others first utilized for gene transfer into neurons in culture and into brains of living rodents [3, 4, 6]. It was then recognized that many viral agents which do not normally cause disease in a particular organ could still function well as efficient gene transfer agents, which is the main goal of gene therapy. This led us to identify the adeno-associated virus (AAV) as a potentially powerful agent for gene transfer into the mammalian nervous system [7]. AAV is not naturally pathogenic in humans, and the small size of this virus permits ready manipulation of the genome such that vectors which contain only the gene of interest packaged into an AAV coat without any viral genes could easily be created and purified without contaminating helper viruses. We first demonstrated that AAV could be an effective agent for safe and long-term gene delivery in the brain, and at that time also showed that transferring a gene for tyrosine hydroxylase, the rate limiting step in the synthesis of dopamine, into the striatum could improve symptoms in a rodent model of Parkinson's disease [7]. That AAV strain, which we now know as AAV serotype 2, also had a particular preference for

neurons, which was unexpected. The combined features of a highly pure vector that results in efficient neuronal gene transfer and does not produce viral proteins, does not provoke inflammation or cell death and leads to long-term, stable gene production made AAV the first agent that could realistically be considered for a first-in-human use for brain gene therapy.

Preclinical Data

The goal of our research program from the start was to translate gene therapy into a human therapeutic. While the advent of AAV technology as a gene delivery vehicle for the nervous system facilitated an enormous amount of experimental research, we sought to identify an early opportunity for human central nervous system gene therapy that could be sufficiently safe and effective in a patient population with an unmet need that would justify proceeding with this novel technology. Our long focus on Parkinson's disease made this a clear favorite, and the rationale outlined above strengthened our interest in developing a human gene therapy for this disorder. Although our initial publication on AAV identified a potential approach, the failure of cell transplants, which were clearly synthesizing dopamine, to reliably improve patients in randomized studies raised some questions as to whether a gene therapy approach would be more likely to succeed [8–11]. Another concern was the location in the brain which would require gene therapy for dopamine replacement. In animals, the target of both cell transplants and tyrosine hydroxylase gene therapy by ourselves and others was the striatum. Since success in animal models failed to translate into success in humans for most prior advanced Parkinson's disease therapeutics, we were concerned that the introduction of too many untested variables in earlier clinical trials may have adversely influenced outcomes. To that point, there was virtually no history of operating in the human striatum. We thus felt that the lack of knowledge as to the proper method for targeting the striatum and potential for heterogeneity of the human striatum could introduce a variable that might confound any study regardless of the potential for effectiveness of the therapy itself.

In order to improve the possibility of success in humans, we began to focus on potential gene therapy opportunities that would be based upon therapies which were already beneficial in human patients. One of the more useful therapies for patients with reduced effectiveness and/or complications of medical therapy is deep brain stimulation of the subthalamic nucleus (STN) [2, 12, 13]. The STN is a key node within the basal ganglia circuitry regulating movement. This is normally controlled by structures which are indirectly regulated by dopamine, such that the loss of nigral neurons leads to STN dysfunction and corresponding abnormal regulation of STN downstream targets [14]. Lesioning of the STN (subthalamotomy) has been found to be effective in human patients, but the longevity of this effect can be limited in some patients and, while most patients requiring surgery have bilateral disease, bilateral lesioning is not well tolerated [15, 16]. STN DBS appears to modulate activity of this structure, and while it remains unclear as to whether DBS

acts by inhibiting neuronal function or modulating neuronal firing patterns, the consequence of DBS is similar to lesioning, with reduction in abnormal brain activity patterns and improvement in clinical motor symptoms. The methodology for targeting the STN, including image-based identification followed by intraoperative target refinement using microelectrode-guided electrophysiological recordings of neuronal activity, is standard for most neurosurgeons actively performing DBS [17]. Therefore, we felt that a gene therapy approach centered upon the STN would be a good candidate for a first-in-human application of in vivo gene therapy in the brain by dramatically reducing many clinical trials variables, since this would be based upon a brain target already shown to effectively improve symptoms in human Parkinson's disease, patients who are good candidates for such procedures are already identifiable and targeting of an infusion catheter would be based upon established surgical methods.

Given the abnormal physiology of the STN in Parkinson's disease, we settled upon the glutamic acid decarboxylase (GAD) gene as the therapeutic agent for AAV-mediated gene therapy in the STN. The GAD gene encodes an enzyme which catalyzes the rate-limiting step in the synthesis of GABA, the major inhibitory neurotransmitter in the brain. Conventional theories regarding basal ganglia circuit dysfunction in Parkinson's disease suggest that loss of GABAergic inhibitory tone into the STN following nigral dopamine cell loss leads to alterations in STN activity [14]. The consequent hyperactive and/or abnormal patterning of STN glutamatergic outflow leads to dysregulation of downstream STN targets, including the globus pallidus interna (GPi) and substantia nigral pars reticulate (SNr). We therefore hypothesized that providing the STN with a novel GABA synthetic capacity through AAV-mediated transfer of the GAD gene would lead to normalization of STN activity, as well as provide increased GABAergic tone to downstream STN targets which are also dysregulated. Additional support for this hypothesis derived not only from prior animal studies with infusion of GABA agonists into the STN, but from human studies which demonstrated improvement not only from lesioning but transient benefit from an acute infusion of the GABA agonist muscimol into the STN of awake Parkinson's disease patients prior to implantation of their planned DBS electrodes [18, 19]. Furthermore, the fact that STN lesioning was an acceptable if suboptimal procedure provided an unusual safety mechanism, such that any potential adverse effect of chronic GAD expression in the STN or from the presence of AAV in that region could theoretically be reversed by lesioning, thereby treating the patient's symptoms and eliminating the source of ongoing gene production.

Prior to entry into human clinical trials, preclinical data was generated to support the hypothesis that AAV-GAD gene therapy in the STN could be safe and effective in Parkinson's disease. We first demonstrated that AAV-GAD gene therapy in the STN could improve abnormal rotations following dopamine agonist treatment which are characteristic of parkinsonian rodents with unilateral 6-hydroxydopamine (6OHDA) lesions of the substantia nigra dopamine neurons [20]. AAV-GAD also improved a variety of spontaneous motor behaviors which may be more relevant to the human condition, including overall locomotor activity, limb use, and head position bias. These results were subsequently supported by an independent group using a similar

approach [21]. In order to confirm the network concept outlined above, we used in vivo microdialysis to measure GABA release in the SNr. Electrical stimulation of the STN to drive STN activity in 6OHDA rats following injection of AAV-GAD led to a significant peak in SNr GABA release which was not observed in animals treated with a control vector [20]. This suggested a potential autoregulatory function, since a greater level of abnormal STN activity would lead to a greater level of GABA release downstream of the STN. Finally, based upon some evidence that STN hyperactivity can cause excitotoxicity and exacerbate nigral dopamine neurodegeneration, we pretreated animals with STN AAV-GAD prior to lesioning and in fact found a significant reduction in nigral dopamine cell loss compared with controls.

Phase I Study

Based upon these results, we developed and proposed a first-in-human trial of AAV gene therapy in the adult brain, with the goal of treating Parkinson's disease patients with AAV-GAD in the STN. Although there was some evidence of disease modification using this approach, the goal of the trials and any eventual therapy was to improve patient symptoms, with any potential for neuroprotection being a secondary possible benefit. To support a filing for a human trial, we completed a study in MPTP primates, which demonstrated long-term safety as well as behavioral improvement in the AAV-GAD animals compared to baseline [22]. In order to provide more reliable physiological data in any human study compared with clinical observations alone, we intended to use positron emission tomography (PET) as a measure of local biological activity in various brain areas at baseline and following treatment. To test this, we used a similar paradigm in these parkinsonian primates and demonstrated significant improvements in abnormal brain metabolism following AAV-GAD compared with baseline and compared with control animals [22].

Following review by both the Recombinant DNA Advisory Committee (RAC) and the U.S. Food and Drug Administration, AAV-GAD in the STN was approved to begin a phase I study in patients with moderate to advanced Parkinson's disease. The study was designed initially to treat both hemispheres of the brain, since most patients who are no longer adequately responsive to medication have disease and symptoms bilaterally. However, since this was going to be the first time that a viral vector would be infused directly into the brain of an adult human for any nonlethal degenerative disorder, there were concerns about the potential for unknown toxicities despite a strong preclinical safety record. It was therefore decided that the phase I study would be an open-label, single-center study of unilateral AAV-GAD infusion into the STN. This was based upon the belief that should an unanticipated adverse event occur, it is less likely to be devastating if it were limited to only one hemisphere of the brain. In order to increase the possibility of observing a clinically meaningful signal, the more symptomatic hemisphere was chosen for treatment, since most patients at this stage still have asymmetry to their disease. Follow-ups of 1, 3, 6, and 12 months were chosen for safety, which was the primary endpoint, as

well as for efficacy analysis, and fluorodeoxyglucose (FDG) PET was chosen as a secondary outcome measure of biological activity. Twelve patients who met clinical entry criteria were enrolled in the study, with the first patient receiving AAV-GAD gene therapy in August, 2003.

The results of the phase I study indicated that unilateral AAV-GAD was safe in the chosen patient population over 1 year, and there was sufficient evidence of effectiveness to justify a more rigorous follow-on study [23]. Subjects demonstrated significant improvements in the motor subsection (part III) of the Unified Parkinson's Disease Rating Scale (UPDRS) over time, with a trend toward improvement at 1 month and significant improvements at subsequent time points. Breaking down the motor scores by body side indicated that most of the overall UPDRS part III effect was due to improvements in motor function of the body side opposite the treated hemisphere, as expected. There was also a suggestion of quality of life improvements and no evidence of toxicity over the course of the study.

PET analysis further confirmed the potential therapeutic benefit of AAV-GAD [24]. As indicated earlier, a consequence of nigral dopamine cell loss is a dysfunction in basal ganglia circuitry, which ultimately leads to abnormal activity of cortical brain regions as well. These can be quantified by FDG-PET, since neurons metabolize glucose proportionate to their level of activity, so uptake of radioactive glucose can be an effective measure of alterations in the activation of neurons grouped in particular brain regions [25]. Using this approach, we and our collaborators were able to demonstrate abnormalities in baseline motor circuitry metabolism in the brain which were significantly improved at 6 and 12 months following treatment with AAV-GAD. Although the clinical evaluations were unblinded and uncontrolled, as is usual for initial phase I trials, the individual analyzing the PET scans was blinded relative to side of treatment. Therefore, the PET studies were controlled, since the untreated hemisphere served as a control for the treated hemisphere, and was single-blinded, since the examiner was unaware of the treatment status of each hemisphere, and since each patient was treated based upon the more severe side clinically and were not uniformly treated in the same hemisphere. Therefore, in addition to a significant improvement over baseline, the improvement was also significant relative to the untreated hemispheres, which did not improve over time.

Phase II Study

The safety and efficacy results of the phase I study were encouraging, and the PET data provided a level of independent biological support that is unusual for a routine, open-label pilot study. Nonetheless, the history of randomized, blinded studies of otherwise promising cell or biological therapies for neurodegenerative disorders which failed to support initial pilot studies created an imperative to proceed with a more rigorous trial [8, 9, 11, 26–28]. Therefore, we developed a multi-center, randomized, double-blind protocol to compare patients treated with AAV-GAD to matched

controls treated with sham surgery. Sham surgery was performed by generating a partial-thickness burr hole in the skull, such that patients would perceive the drilling and the cap for the infusion system (see below) would be inserted, but the inner table of the skull would not be violated, thereby eliminating the risk of intracranial injury for control patients. A 6-month blind was chosen as the primary outcome, in order to reduce the time that patients would need to stay blinded, since maintaining a blind for a long period can be difficult, with the plan to follow patients for a full 12 months as part of the ongoing safety and efficacy analysis. The trial was 1:1 design, so that a relatively equivalent number of patients were randomized to each group, in order to enhance the statistical power of the study, with roughly 40 patients planned to be enrolled overall. All patients and caregivers remained blinded until the final patient reached 6 months after treatment, in order to prevent bias being introduced from serial unblinding. Although this meant that some patients were blinded for longer than 6 months, the enrollment was sufficiently robust that a relatively small number of patients reached 12 months or beyond in the blind.

One difference in this study compared with the phase I trial was the plan to treat patients bilaterally, given the likely need for bilateral surgery among most patients who might eventually be candidates for the therapy should it reach approval. Another change was based upon the concern over patient variability. While centers in our trial and in other studies were chosen based upon a track record of expertise in this area, one potential confound that could lead to great variability between studies is the confidence of the clinical diagnosis for patients entering the study. Since even multi-center studies are necessarily much smaller for neurosurgical interventions compared with drug trials, only a small number of patients who meet entry criteria but turn out not to have the clear disease pattern can destroy the statistical power of a study testing an otherwise promising therapy. Therefore, we used FDG PET as an entry criteria for the study. Patients did not have to meet a particular level of abnormality on PET, but their pattern of abnormal metabolism needed to be within established criteria from earlier PET studies in Parkinson's disease [25, 29, 30]. This in fact resulted in exclusion of several patients who might have otherwise clinically met criteria for enrollment.

Another substantial change from phase I to phase II was the method for infusing AAV-GAD into the STN. In phase I, we adapted a method that we had previously used for in vivo microdialysis in human patients, using a borosilicate glass fiber as an infusion catheter passed through a microelectrode guide tube, and attached to an external pump, with infusions were completed in the operating room. For the randomized study, we developed a system that might be more amenable to general clinical use. The catheter was flexible so that it could reside in the brain following removal of the guide tube without causing local trauma, similar to a DBS electrode. The last 1 cm tip of the catheter was steel, which would not absorb AAV based upon our testing and which was visible on CT scans. Again similar to the DBS electrode, a locking cap was created to lock the catheter in place to prevent migration during the infusion. A system for releasing the catheter was also created, so that the infusion could take place outside of the operating room following catheter insertion, with the catheter then removed at the bedside after infusion was completed without

necessitating a return to surgery. Since this was an untested system, there was the possibility that a catheter failure or migration could lead to poor or off-target infusion which could limit efficacy. Therefore, the protocol specified that only patients with documented bilateral catheter placements within a predefined zone considered to include the STN, and with greater than 50 % infusion of the vector fluid volume with confirmation of catheter patency following removal, would be included in the per-protocol analysis. Determination of catheter location was made by an expert DBS surgeon who did not perform a procedure in the study and who analyzed all of the post-infusion CT scans while blinded, without knowledge of patient outcome, prior to data analysis. Sham patients also underwent infusion of saline into the partial thickness burr hole. CT scans were either performed under an alias or were noted as study images and were not included in the main PACS system, to minimize the risk of unblinding. A blinding questionnaire performed throughout the study duration suggested that the blinding procedures were effective.

The results of this study demonstrated that AAV-GAD in the STN was effective compared both to baseline and compared with the contemporaneous sham surgery group [31]. Interestingly, as has been observed in the past, the sham group did show a significant improvement relative to baseline as well, but the AAV-GAD group had roughly twice the improvement on the UPDRS part III motor scores compared with controls. This represented the first demonstration of a significant improvement for a biological or cell-based therapy in the brain compared with a contemporaneous sham surgery control group, indicating that gene therapy can in fact be effective for neurodegenerative disorders. The magnitude of the effect was similar to the effect size in a recent large national U.S. study of DBS compared with best medical therapy, although it was somewhat less than European trials of the same therapy. There was also a significant improvement in the number of hours spent in the better or "ON" state at 3 months following surgery and a strong trend towards improvement at 1 month. While this was not significant at 6 months, likely due to variability in patient diaries in a small study, there again was a significant difference between groups at 12 months (data not shown). There was also evidence of a decrease in complications of medical therapy, with a significant improvement in the UPDRS part IV scale of therapeutic complications at 6 months relative to baseline and a trend at 3 months, with no change in control patients over that time period. Finally, there were no complications related to the gene therapy over the course of the study. The infusion device did fail in some patients, leading to exclusion from the per-protocol analysis, but a subsequent small design change appears to have addressed this issue.

FDG PET performed prior to and following treatment provided an opportunity for further exploration into the biological basis for these findings. There was evidence of improvements in particular PET patterns in treated patients compared with controls, and a pattern was identified which uniquely correlated with clinical improvements in AAV-GAD patients compared with controls (data not shown). One interesting observation was an analysis of PET patterns which could discriminate true treatment responders from sham or "placebo" responders. To do this, we and our collaborators analyzed the FDG-PET scans from sham surgery patients at the

end of 6 months, prior to unblinding, and compared these with baseline, to identify patterns that were common to sham responders as compared with either AAV-GAD responders or sham non-responders. Using unbiased mathematical modeling, a pattern was identified consisting of brain regions associated with affect and mood, which was termed the sham surgery-related pattern [32]. This pattern was generated from half of the sham surgery responders. A prospective analysis of the remaining half confirmed that this pattern was present in that group as well. This pattern was not present in sham non-responders, nor was it present in the AAV-GAD treatment responders, further suggesting that the AAV-GAD treatment response was a genuine biological effect distinct from the sham response.

Summary

The potential for gene therapy as a direct means of exploiting the power of genetic research has been and remains quite promising for a variety of neurological diseases. The development of AAV technology has greatly facilitated the potential for translating gene therapy into a successful human treatment, with many studies now having been completed or underway for diseases including Parkinson's disease, Alzheimer's disease, and various lethal pediatric neurogenetic disorders. The safety and suggestion of efficacy of AAV-GAD in the first phase I human trial provided support for many of these approaches through the demonstration of the possibilities of AAV technology in the human brain. Although as of the writing of this contribution, further studies of AAV-GAD to support final regulatory approval are in the planning stages, the success of AAV-GAD as the first CNS gene therapy to show efficacy compared with a sham group in a gold-standard randomized, double-blind clinical trial has provided and should continue to provide support for the future development of gene therapy as a useful treatment for neurological disorders.

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Chapter 6
Antisense Oligonucleotides for Amyotrophic Lateral Sclerosis

Wade K. Self and Timothy M. Miller

Abstract Antisense oligonucleotides (ASO) are short DNA-like chemicals that bind to RNA by Watson–Crick base pairing and modulate function of the RNA. These chemicals do not cross the blood brain barrier, but may be delivered directly to the cerebral spinal fluid (CSF) to achieve widespread distribution throughout the brain and spinal cord. ASO have been used to target genes associated with familial amyotrophic lateral sclerosis (ALS), such as SOD1 and C9orf72 as well as miRNAs. A Phase I trial for SOD1-targeting ASO showed excellent safety and important pharmacodynamics. ASO are a promising therapeutic approach for ALS.

Keywords ALS • SOD1 • C9orf72 • mRNA • miRNA

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by progressive weakness, muscle atrophy, difficulty breathing and swallowing secondary to loss of motor neurons in the brainstem and spinal cord [1]. Of the approximately 30,000 people in the United States that live with ALS, most die from respiratory failure within 3–5 years post diagnosis. Currently, one FDA-approved medication, Riluzole, for ALS exists that slows disease progression, but the small magnitude of the effect on survival (3–6 months) [2] is disappointing and underscores the urgent need for novel therapeutic approaches to ALS. One potential reason for the lack of efficacy in recent ALS clinical trials may be due to the well-known heterogeneity of the disease. This recognition combined with new targeted therapeutic tools has sparked interest in identifying subsets of ALS patients for whom particular therapies might be most appropriate. Dominantly inherited ALS may represent such a subset. 5–10 % of ALS is familial, with known mutations accounting for 50–60 % of these cases. Targeted therapeutics strategies are being developed for the most common familial forms of ALS: *SOD1* and *C9ORF72*, as well as miRNAs implicated in disease. One promising therapeutic strategy that has

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already been translated from animal models to clinical trial in ALS is the use of antisense oligonucleotides [3].

Antisense oligonucleotides (ASO) are single stranded, short (typically 20 mer), modified DNA-like chemicals that bind to a specific RNA target sequence by typical Watson–Crick base pairing (reviewed in [4]). The chemical modifications of the ASO increase its stability in biologic fluids, increase the potency of binding to the target, and particular modifications of the ASO influence the effect of the ASO-RNA duplex in the cell. A typical ASO modified at the 2′ position on the first 5 and last 5 nucleotides without modifications in the center 10 nucleotides will activate RNAse H activity in the nucleus leading to catalytic degradation of the targeted mRNA. ASO that are fully modified at the 2′ position do not activate RNAse H activity and may be used to change splicing activity or to inhibit miRNAs (Fig. 6.1). These diverse mechanisms of ASO incite promise in their application as therapies in neurodegenerative diseases with a broad range of disease mechanisms. In a disease with a heterogeneous population such as ALS, the diverse mechanisms by which ASO can target mRNA are particularly attractive. Delivery of ASO to the CNS was first considered in the context of targeting SOD1 and has progressed to clinical trials. Subsequent studies have identified new targets where ASO therapy may be effective in ALS, including a repeat expansion found in the first intron of the C9ORF72 gene, as well as microRNAs implicated in ALS disease progression.

SOD1

In 1993, Rosen and colleagues identified that mutations in the gene encoding superoxide dismutase 1 (SOD1), a homodimeric metalloenzyme that catalyzes the conversion of superoxide anion to hydrogen peroxide and molecular oxygen [5], cause dominantly inherited ALS with high penetrance in approximately 15 % of familial cases [6]. In addition to motor neuron loss, a distinct pathology in tissues of SOD1 ALS patients is the presence of misfolded, insoluble SOD1 protein aggregates within the central nervous system [7]. Two decades of work in animal models harboring mutant human SOD1 transgenes and the dominantly inherited nature of the disease all suggest a toxic gain of function resulting from the accumulation of misfolded mutant SOD1 protein in the central nervous system (reviewed in [8]). The toxic gain of function hypothesis is further supported by the observations that SOD1 knockout mice display a relatively mild motor phenotype, and that SOD1 heterozygote mice are normal [9]. These studies also suggested that reduction in SOD1 is safe and tolerable. Therefore, knockdown of mutant SOD1 at the mRNA and protein level presented as an attractive therapeutic approach to prevent motor neuron loss in ALS patients harboring SOD1 mutations.

With this strategy in mind, Miller, Smith, and colleagues developed ASO that decreased SOD1 in rats, but also demonstrated that these ASO would not cross the blood brain barrier [10]. Since ASO are delivered without virus or any carrier, the lack of blood brain barrier penetration by these highly charged molecules was predicted.

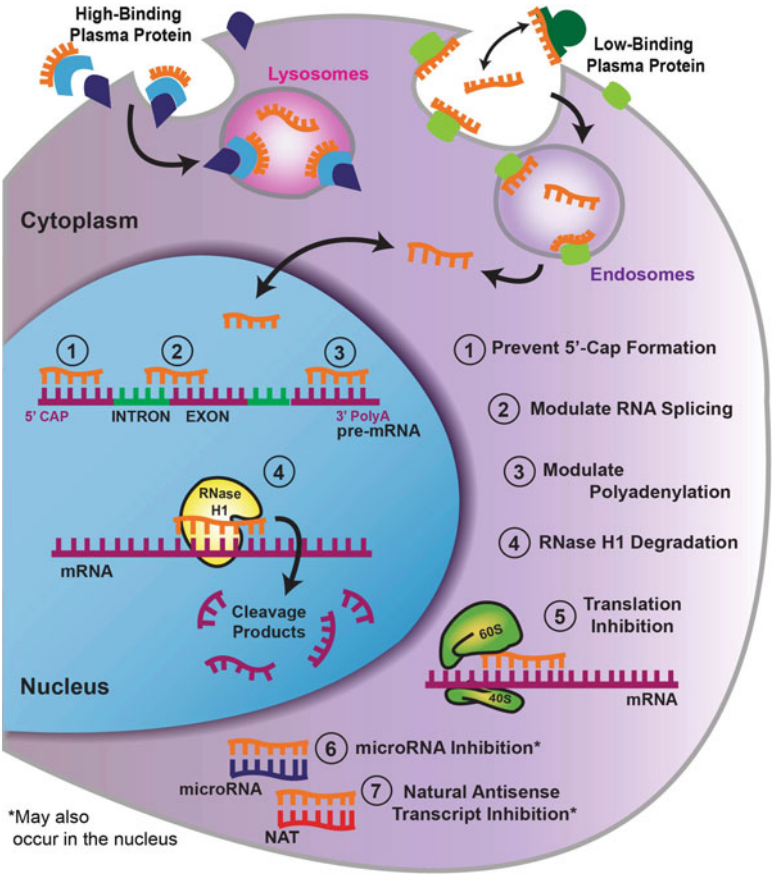

Fig. 6.1 Antisense oligonucleotide (ASO) mechanisms of action. ASO have been proposed to enter into cells through high- and low-binding plasma protein receptors on the cell surface, resulting in ASO compartmentalization into lysosomes and endosomes. Through a largely unknown mechanism, ASO are released from the vesicles into the cytoplasm where they can freely move in and out of the nucleus. Upon entry into the nucleus, ASO can bind directly to mRNA structures and prevent the formation of the 5′-mRNA cap (1), modulate alternative splicing (2), dictate the location of the polyadenylation site (3), and recruit RNaseH1 to induce cleavage (4). ASO in the cytoplasm can bind directly to the target mRNA and sterically block the ribosomal subunits from attaching and/or running along the mRNA transcript during translation (5). ASO can also be designed to directly bind to microRNA (miRNA) sequences (6) and natural antisense transcripts (NATs) (7), thereby prohibiting miRNAs and NATs from inhibiting their own specific mRNA targets. Ultimately, this leads to gene upregulation of the miRNA and NAT targets (Reproduced with permission, Neurotherapeutics (2013) 10:486–497)

Since therapeutics including chemotherapy and pain medications are routinely delivered directly to the cerebral spinal fluid (CSF) in humans, direct delivery of ASO to the CSF was attempted. Surprisingly, the ASO were distributed throughout the brain and spinal cord [10]. This remarkable distribution of the ASO was confirmed in

subsequent studies targeting huntingtin in both mice and nonhuman primate [11] and tau in mice [12]. Distribution data are not yet available from human trials. The empiric distribution data suggest an active uptake mechanism for these ASO rather than simple diffusion because the ASO are seen deep within the CNS without a particular noticeable gradient from CSF spaces. The initial rat studies for SOD1 focused on intraventricular delivery while the human studies for SOD1 used intrathecal delivery. With excellent safety data in humans for intrathecal delivery and excellent distribution seen with huntingtin in nonhuman primates with intrathecal delivery, it is likely that intrathecal delivery will be the method of choice for neurodegenerative syndromes, including subsequent studies in ALS. However, an intraventricular approach remains a viable option. Intraventricular ASO delivery in rats expressing human SOD1 demonstrated widespread delivery of the ASO as well as decreased levels of SOD1 mRNA and protein. This reduction in mRNA and protein also prolonged survival in the SOD1 rat animal model [10]. The promising results in these animal models and subsequent toxicology studies in both rats and nonhuman primates supported application of these ASO to humans with mutations in the SOD1 gene.

At the time of the start of the SOD1-focused ASO, there was no previous experience with using this chemical class delivered to the CSF, though there was ample experience outside of the CNS [13]. Based on this first-in-man approach, the first CNS ASO trial used one dose of a small amount of ASO [3]. Unlike typical Phase I trials that may be conducted in a healthy volunteer population, this study was conducted in patients with symptomatic ALS and known SOD1 mutations. There were four dosing cohorts, each containing six on drug and two on placebo, with each subsequent cohort at a higher dose. There were 21 individual participants, some of whom participated in more than one cohort, for a total of 32 doses. Overall, the ASO was well tolerated with no findings attributed to the ASO. There were, however, adverse events in the clinical trial. About a third of participants, both in placebo and ASO treated groups, had a headache and/or nausea for 1–2 days that was markedly worsened by standing up and resolved nearly completely with lying down, a characteristic syndrome for low CSF pressure. Given the placement of an intrathecal catheter for 12 h to deliver the ASO (or placebo), this type of syndrome was not surprising. In approaching the FDA for an Investigator New Drug (IND) application needed to conduct the clinical trial, one of the major unknowns was the pharmacokinetic properties of the ASO chemical class delivered to the CSF. Understanding the pharmacokinetics allows for predictions of how a particular dose in humans will correlate with observed toxicities in animal studies. Thus, one of the major accomplishments of this first CNS ASO trial was to better understand pharmacokinetics in the CSF and to test prediction models used to make the links between animal toxicology studies and doses in humans. As shown in Fig. 6.2, the observed ASO levels in the serum and CSF were close to the predicted values. These data will be incorporated into future clinical trials of ASO. The small dose of ASO used in this first trial did not affect SOD1 levels, but the ability to demonstrate a pharmacodynamics effect will be an important component of the next clinical trial.

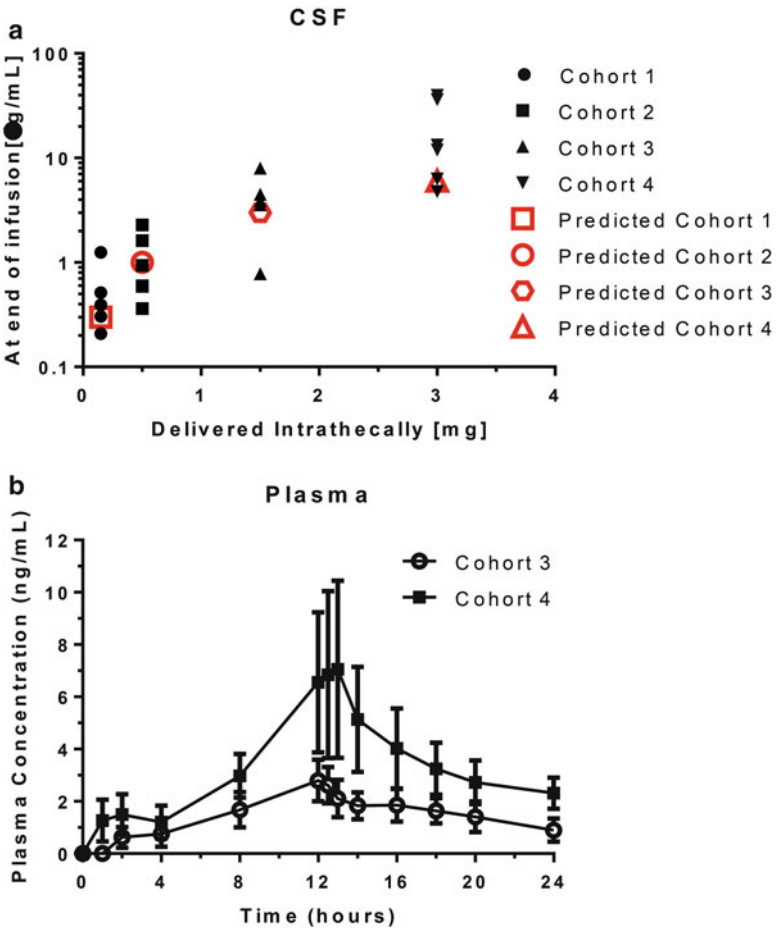

Fig. 6.2 CSF and Plasma Concentrations of Isis336111. (**a**) CSF was drawn immediately after the end of the intrathecal infusion (11 h, 22 min) one level above or below the infusion site. Measured ISIS333611 concentrations and predicted values are shown. (**b**) Plasma was drawn at each of the indicated time points for all four cohorts and ISIS333611 measured by ELISA. As anticipated, plasma levels for cohorts 1 and 2 were below the limit of detection of the assay ($N = 6 \pm$SE) (Reproduced with permission, Lancet Neurol. 2013 May; 12(5):435–442)

C9ORF72

In 2011, a GGGGCC hexanucleotide repeat expansion in the noncoding region of the *C9ORF72* gene was discovered to be the leading genetic cause of both ALS and frontotemporal dementia [14, 15]. This repeat expansion is present in ~6 % of sporadic ALS patients and in 40 % of familial ALS patients [16]. Similar to SOD1, studies suggest a gain of toxicity function associated with the repeat expansion, as no coding mutations have been found in the *C9ORF72* gene [17], discouraging a loss of

function of C9ORF72 resulting in disease. The function of the C9ORF72 protein, however, remains unknown. These repeat expansions result in two distinct pathologies that are independent of C9ORF72 protein translation in patient tissues: nuclear repeat expansion RNA foci and repeat-associated non-ATG (RAN) translation of insoluble polypeptides from these repeat expansion transcripts [18, 19]. Although still debated, studies suggest that both the formation of RNA foci and RAN polypeptides are toxic to neurons in cell culture [20] and in vivo drosophila models [21, 22], emphasizing the need for therapies targeted towards the gain of function toxicity associated with this hexanucleotide repeat expansion.

Because the repeat expansion is located in the noncoding region of *C9ORF72*, ASO-mediated therapies provide an attractive option in mitigating toxicity associated with RNA foci and RAN translation polypeptides. Multiple studies have utilized an ASO strategy in cell culture to demonstrate that, unlike siRNA strategies that target coding regions of the gene, ASO can be specifically designed against the intronic repeat expansion to prevent the formation of RNA foci in fibroblasts [23], induced pluripotent stem cell (iPSC)-derived heterogeneous neuron cultures [24], and iPSC-derived motor neurons [25] from patients harboring the repeat expansion. Additionally, the levels of C9ORF72 protein remain unchanged in these culture models upon ASO treatment, validating that the ASO specifically inhibits the repeat expansion associated with neurotoxicity. Patient-derived iPSCs have been invaluable for initial screening of the therapeutic efficacy of compounds targeting the hexanucleotide repeat expansion, including ASO, as directly converted cells harbor the repeat expansion that is technically difficult to generate using conventional cloning techniques and exhibit the pathology observed in patient tissue samples. These models, however, fail to recapitulate the toxicity associated with these repeat expansions. Although iPSC-derived neurons with the repeat expansion display electrophysiological phenotypes of diminished capacity to fire continuous action potentials [25] and susceptibility to excitotoxicity [24], these cells fail to degenerate in culture due to these repeat expansions. Despite the absence of a degenerative phenotype, the ASO administered to the iPSC-derived neurons were successful in restoring physiological function similar to control iPSC-derived neurons without repeat expansions. Another caveat of this system is the inability to study the pharmacokinetic distribution of ASO upon delivery to the CNS. Thus, it will be crucial to test candidate ASO in animal models to not only assess the ASO mechanism of action, but to ensure that each drug will be delivered to the correct cell types in the CNS.

In the absence of an animal model that expresses the repeat expansion in *C9ORF72*, Lagier-Tourenne and colleagues investigated the efficacy of a C9ORF72 ASO in a non-transgenic mouse model [23]. The group showed that delivery of the ASO via intracerebroventricular injection to knock down expression of C9ORF72 protein is tolerated in normal mice, supporting that loss of C9ORF72 function is not the cause of neurodegeneration in repeat expansion carriers. Data from C9orf72 knockout mice will further inform on this result. Importantly, these results also suggest silencing of the entire *C9ORF72* gene is still a potential therapeutic option in ASO design, similar to *SOD1*. Also, the group demonstrated that the ASO is broadly distributed throughout all cell types of the brain and spinal cord. These data provide promise for future application of a C9ORF72 ASO as a potential therapy for hexanucleotide repeat

expansion carriers. The development of transgenic animal models that express the *C9ORF72* gene with the repeat expansion will be important in assessing whether these ASO will be effective in ameliorating the neurotoxic phenotype associated with this expansion in humans, similar to previous studies performed in transgenic animal models expressing mutant SOD1.

Contrary to the approach of targeting the coding portion of a gene, as seen in ASO to SOD1, hexanucleotide repeat expansions seen in noncoding regions of *C9ORF72* present unique challenges when considering design of targeted ASO therapies. Transcription of noncoding DNA with the hexanucleotide repeat expansion results in both a sense strand transcript $(GGGGCC)_n$ and an antisense strand transcript $(GGCCCC)_n$. Both sense and antisense transcripts are present in RNA foci [24, 26], and also undergo RAN translation. Depending on the frame by which translation is initiated, multiple RAN translation repeat polypeptides can be generated in cells: Glycine-Alanine $(GA)_n$,, and Glycine-Arginine $(GR)_n$ from the sense transcript, Proline-Arginine $(PR)_n$ and Alanine-Proline $(AP)_n$ from the antisense transcript, and Glycine-Proline $(GP)_n$ from both transcripts. Although still debated, studies suggest that arginine-containing polypeptides $(GR)_n$ and $(PR)_n$ are neurotoxic [21, 22], which result from translation of both sense and antisense transcripts. Taken together, these data suggest that the most effective design for an ASO-mediated C9ORF72 treatment must target both the sense and antisense repeat expansion transcripts. This hypothesis is supported by the fact that, despite treatment of ASO to the sense strand that inhibit RNA foci, antisense polypeptides are still present in fibroblasts and iPSC-derived neurons after drug administration [23, 24]. These observations further emphasize the need for an adequate neurotoxicity model to test the efficacy of ASO's, as sense-autonomous neurodegeneration may occur when one transcript is silenced. Despite the current promise of designing ASO that effectively target non-coding repeat expansions in a gene without affecting the expression of coding mRNA, the application of such ASO for the treatment of repeat expansion carriers in the *C9ORF72* gene remain in early development.

miRNAs and ALS

microRNAs are short (typically 20 nucleotides) RNAs that regulate the translation of mRNAs. They are recognized as an integral part of a cell's translational control and have been implicated in many disease processes, including ALS [27]. Because miRNAs regulate many mRNAs, there has been some concern about miRNAs as therapeutic targets, yet inhibiting one particular miRNA (and thus potentially affecting many mRNAs) has not only been well tolerated in humans, but was impressively effective. Based on the observation that Hepatitis C requires miR-122 for viral replication, ASO inhibitors of miR-122 were developed as therapeutics for Hepatitis C. In a recent Phase II trial, treatment with an ASO that inhibits miR-122 showed a marked reduction of Hepatitis C serum viral load [28]. Thus, targeting one miRNA has great therapeutic potential. The challenges for application of this approach to neurodegeneration are determining the key miRNA changes in a syndrome and developing CNS targeting strategies for miRNA therapeutics.

In ALS, Koval and colleagues screened miRNA changes in SOD1G93A mouse model of ALS and found 12 miRNAs that were increased [29]. Six of these miRNAs were also increased in human, postmortem ALS spinal cords compared with controls. Surprisingly, both familial and sporadic ALS showed the same miRNA changes, suggesting a possible ASO-mediated treated for a larger ALS population. In order to determine which miRNAs are important for ALS, Koval and colleagues first tested whether ASO that target miRNAs would distribute as broadly to the CNS as do ASOs. Indeed, these ASO also showed excellent distribution throughout the brain and the spinal cord. Ideally, to test the importance of each of the defined changes in miRNAs in ALS, each one of these miRNAs would be inhibited in the mouse model to determine the effect on disease pathogenesis, as some increased miRNAs may be compensatory, beneficial changes. Koval and colleagues studied miR-155 and found that inhibition of miR-155 slowed progression in the SOD1G93A disease model. The fact that miR-155 inhibition delayed disease progression in SOD1G93A mice was later confirmed by a separate study [30]. These two studies have increased enthusiasm for miR-155 as a therapeutic target in ALS. The fact that one study showed increased miR-155 in peripheral blood mononuclear cells (PBMCs) in patients with ALS [30] suggests that increased miR-155 in PBMCs could be used as part of enrollment criteria for a miR-155 inhibitor trial. However, these data must be repeated by others in a larger cohort of ALS vs. controls. Other miRNA changes in ALS have not been tested for how or whether they affect disease progression. It is also possible that inhibition or overexpression of multiple miRNAs may be efficacious, though each miRNA will likely be tested singly in clinical trials.

Pharmacodynamics Biomarkers for ASO in ALS

A difficult challenge in clinical trials is identifying a pharmacodynamics biomarker to assess the therapeutic efficacy of the treatment tested. One advantage of an ASO, mRNA-lowering therapeutic approach is that the pharmacodynamics biomarker may be relatively straightforward to identify. In many neurodegenerative diseases, proteins and miRNAs implicated in disease pathogenesis are present in the CSF of patients. With an ASO-mediated mRNA reduction, one may hypothesize CSF levels of the ASO protein target would decrease, providing an excellent biomarker for the mechanism of action of the ASO within the CNS. Indeed, Winer and colleagues demonstrated this concept in the G93A SOD1 rat model, as a reduction in CSF mutant SOD1 was observed in rats administered an SOD1 ASO compared to saline-treated control animals [31]. The group also observed that SOD1 CSF levels in humans are stable over time, including patients harboring SOD1 mutations. SOD1 protein levels in SOD1 patients were stable over the disease time course. Taken together, these data provide great promise for the use of CSF SOD1 protein levels as a reliable pharmacodynamics biomarker for an ASO therapeutic strategy.

CSF protein levels may also be a dependable pharmacodynamics biomarker for C0ORF72-targeting therapies since detectable RAN polypeptides are present in ALS patients harboring the hexanucleotide repeat expansion, but are absent in patients that do not carry the repeat expansion [32]. However, an important consideration would be the selection of the proper polypeptide or combination of polypeptides to use in such an analysis, as five polypeptides are produced from the sense and antisense repeat expansion transcripts. With current assays, $(GP)_n$ products are the only products found in CSF [32], but further investigation must determine if other RAN polypeptides are present as well. In a similar strategy, miRNA CSF levels have similar potential to be utilized in an ASO treatment targeting specific miRNAs, as miRNAs are present extracellularly in CSF [33].

Although SOD1 and C9ORF72 polypeptides are present in CSF, an important consideration before planning a pharmacodynamics study that modulates CSF protein levels is the protein half-life, which is currently unknown in humans. These values could be determined with the use of stable isotope labeling kinetics in both humans and animal models to calculate the half-life of the protein of interest similarly to quantifications of the amyloid-beta peptide half-life in human patients with Alzheimer's Disease [34, 35]. Additionally, it will be important to understand the correlation between half-life of the protein within the CSF compared to CNS tissue in order to properly understand the efficacy of ASO treatment within the brain and spinal cord as measured by CSF protein levels.

Conclusion

Now that ASO have moved from animal models into a completed Phase I trial, the technology is ready for use for multiple therapeutic targets in ALS. Theoretically, the ability to relatively quickly generate on-target drugs for a particular pathway could greatly accelerate time from target discovery to clinical trial. An added advantage of this type of targeted method is the ability to quickly define a marker for pharmacodynamics. Gathering more safety data and a better understanding of pharmacodynamics will be the key components which determine whether the promise of ASO become a reality for ALS treatment. While applicability to aggressive genetic forms of the disease are likely the best place to start, each of the ASO experiences will be critical steps towards applying ASO to a wider group of ALS patients.

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Chapter 7
Gene Therapy for Inborn Errors of Metabolism: Batten Disease

Dolan Sondhi, Ronald G. Crystal, and Stephen M. Kaminsky

Abstract The development of a gene therapy for inborn errors of metabolism is a multifaceted challenge that rides on organizational, financial, and scientific issues. Using our experience with developing a gene therapy strategy for Batten disease [late infantile neuronal ceroid lipofuscinosis (LINCL), CLN2 disease], these factors are described in the context of the: (1) development of a therapeutic concept for a target disease; (2) pathway to proof of concept via preclinical studies; (3) translation to clinical development; (4) funding and the associated restrictions; (5) assembly of the clinical team; (6) regulatory and compliance requirements; and (7) the iterative process of using lessons learned to inform the next generation therapy. Our experience with each of these factors is demonstrated from our development and clinical translation for two generations of drug product applied to this fatal childhood disorder. Outlined are the descriptions of the hurdles encountered and our solutions, which should be informative for those who seek to develop a gene therapy for a rare disease.

Keywords Batten disease • AAV vectors • Gene therapy • Central nervous system • Translational medicine • Preclinical development • Lysosomal storage disorders • Phase I clinical trials in academic setting • Experimental therapies

Introduction

The blood–brain barrier is a highly selective permeability barrier that limits the diffusion of molecules circulating in blood from reaching the extracellular fluid bathing the brain [1]. Maintained by tight junctions in brain endothelium, the blood–brain barrier prevents proteins in plasma from reaching brain cells. While this barrier protects the brain, it hinders the effectiveness of systemic administration of therapeutic proteins for treating CNS disorders. This presents a challenge in

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treating the CNS manifestations of many inborn errors of metabolism, disorders for which protein therapies are highly effective in treating other organs [2]. One solution to this dilemma is gene therapy, a therapeutic modality where the gene is used as a protein delivery strategy. If the gene can be transferred to cells in the brain, and can effectively produce the protein deficient in a given hereditary metabolic disorder, and can do so on a persistent basis, the blood–brain barrier can be circumvented, and the CNS manifestations of the disorder can be effectively treated.

In this chapter, we discuss our pathway to clinic for gene therapy for a disease with CNS manifestations of an inborn error of metabolism, late infantile neuronal ceroid lipofuscinosis (LINCL), often referred to as "Batten disease" and more recently as "CLN2 disease" based on the affected gene [3]. This path started with brainstorming sessions to identify a target disease and then proceeded through the choice of a gene therapeutic strategy, the design of preclinical experiments, safety and toxicology studies, clinical study design, regulatory hurdles, building a study team, and executing clinical trials. As part of this development program, we encountered the challenges of identifying sources of funding made all the more difficult given a backdrop of motivated families with all of the emotional ties associated with having children with a fatal genetic disease. The opportunity of using the lessons learned was that continuous improvements in the understanding of disease biology and therapeutic design inform the subsequent iterations of a clinical program now enhanced by a newly updated state of knowledge. This strategy led us to locking in on a first generation gene therapy vector as a mechanism of drug delivery in our initial clinical study. Later a second vector provided improved efficacy, as measured by new research tools (an animal model of LINCL) only available after the first trial began and formed the basis of our second clinical trial for the same indication.

In the following text, we present the strategies as they evolved in our developmental pathway. We initiated our LINCL gene therapy program in 2001, the first clinical trial (with AAV2) in 2004, and the second clinical trial (with AAVrh.10) in 2009.

Identifying the Target Disease

The path to our studies was directed by the larger goal of establishing a platform technology for a gene transfer therapy for CNS-based lysosomal disorders with possible extension to the broader range of rare genetic diseases. As an initial step, we defined a set of parameters that we believed were important, in a practical sense, to facilitating the work. These included the severity of the disease which would impact the risk/benefit ratio for testing an experimental therapy, understanding of the molecular basis of the disease with a knowledge of the effected gene and protein, a therapeutic that takes advantage of bystander correction mediated by the gene product and did not require the rigors of regulatory requirements for testing in a nonfatal disease, and the available funding options. For the above stated reasons, we narrowed our focus to LINCL, a lysosomal storage disease which affects the CNS and the eye.

LINCL is an autosomal recessive lysosomal storage disease that primarily affects the CNS. It is caused by mutations in the CLN2 gene, resulting in a deficiency in the levels of the lysosomal enzyme tripeptidyl peptidase I [4, 5]. The result is an accumulation of storage materials in lysosomes, leading to progressive cell death. Clinically, this is associated with progressive neuro- and visual-degeneration starting at the age of 2, with death by 8–12 years [3, 6–9] (Fig. 7.1a). Without any available cure and just palliative therapy, children affected by LINCL and their families have no medical options to improve quality of life [10, 11].

LINCL met all of our prerequisites for development of a gene therapy strategy. It is a fatal disease of childhood, providing a risk/benefit ratio in favor of clinical development, despite the theoretical risk. LINCL meets the definition for an orphan drug target. The disease mostly manifested in the CNS and eye providing a good match for the clinical expertise of our team. The causative gene was known and the

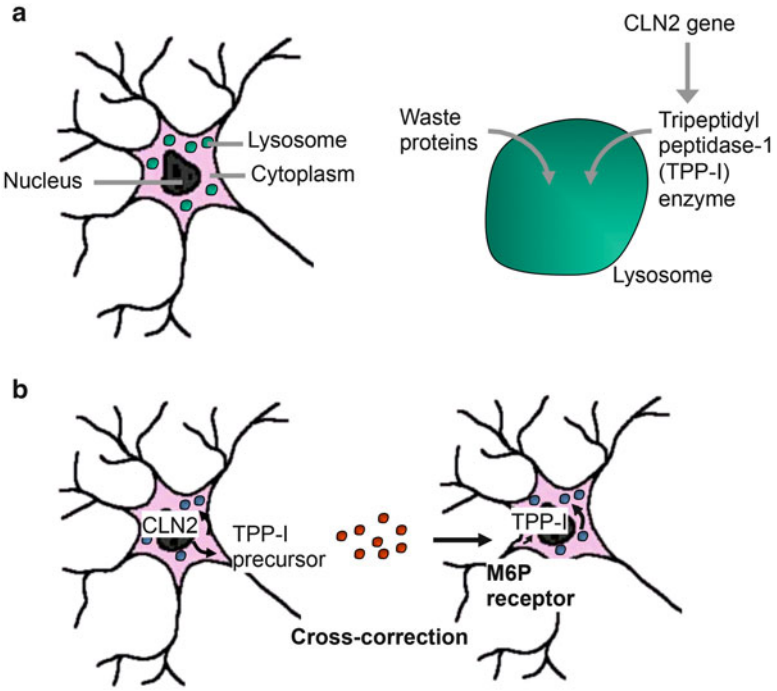

Fig. 7.1 Schematic of tripeptidyl peptide 1 (TPP-I) function within the neuron and leveraging the biology of TPP-I biosynthesis to strategize gene therapy. (**a**) Representation of a neuron with the lysosomes in the cell body (*left panel*). The lysosome functions to degrade proteins into composite amino acids using, in part, the CLN2 gene product TPP-I (*right panel*). (**b**) Cross-correction of neighboring cells by gene therapy. The transduction of the neuron on the *left* with the coding sequences for CLN2 results in expression and secretion of the TPP-I precursor protein which is internalized by the mannose 6 phosphate receptor on neighboring cells. The low pH of the endosome containing the precursor TPP-I cleaves the protein and results in an active peptidase

size of the coding sequences is compatible with viral vector payload limitations in the vector tool kit [4]. Enzyme augmentation therapy would require the repetitive opening of the blood–brain barrier or repetitive administration to a reservoir within the CNS, whereas gene therapy would require a single administration. The gene product is a secreted protein with the capacity to correct neighboring cells using the mannose-6-phosphate receptor pathway, obviating the need to genetically correct every cell in the CNS [12] (Fig. 7.1b). A therapeutic response would likely require only a small fraction of the normal levels of the protein. Finally, there was a highly motivated parent-based foundation willing to provide funding.

Developing the Gene Therapy Approach

We started with the knowledge that we were dealing with a disease that was monogenic, diffuse throughout the CNS and the eye, and its effects were cumulative. The ideal therapy is needed to complement the insufficient amounts of functional gene product, be widespread, and provide sustained treatment. One important issue early in the development phase was whether we were going to treat the CNS only, the eye only or both? We decided to focus on the CNS only, as the deterioration of the CNS dominates the clinical progression and is responsible for the death of children.

These considerations immediately eliminated several possible gene delivery strategies such as nonviral vectors (due to the low efficiency and transience of gene expression) and lentivirus and herpes simplex virus vectors (due to concerns for safety issues). Since the vast majority of the target cells in the CNS do not proliferate, the gene transfer vector had to be capable of effectively transferring a gene to quiescent cells. In addition to safety issues, this constraint eliminated conventional retrovirus vectors based on the Maloney murine leukemia virus, a virus that requires proliferating cells for efficient gene transfer [13]. Of the many vector types in use at the time, the adeno-associated virus (AAV) had all the suitable characteristics required for CNS gene transfer. AAVs have appropriate tissue tropism for CNS, long-term expression, excellent safety profile, packaging capacity compatible with the CLN2 coding sequences and regulatory elements. AAV vectors are easy to construct and are produced in a replication incompetent and recombinant form [14].

Vectors

The vector of choice, AAV, is a naturally replication-defective virus which depends on adenovirus (Ad) or herpes simplex virus for replication. It is a small non-enveloped icosahedral parvovirus with a 4.7 kb single stranded DNA genome [15, 16]. There is no known pathology from wild-type AAV infections. To make recombinant AAV vectors, all viral genes are replaced by an expression cassette for the transgene, leaving intact essential cis elements of the genome, the inverted

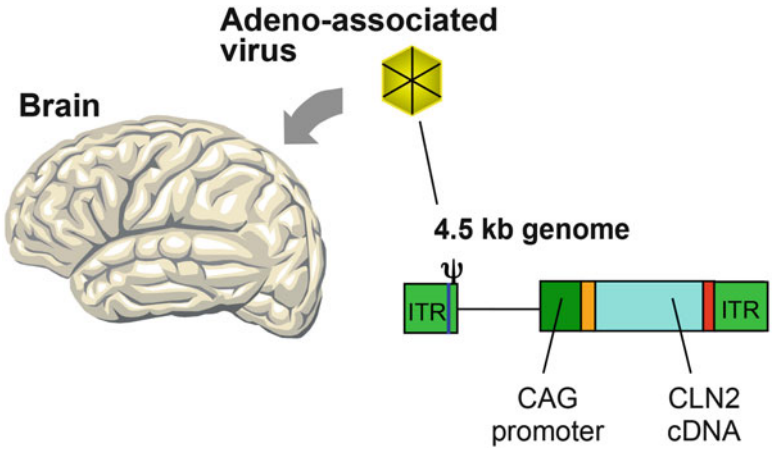

Fig. 7.2 AAV vector expressing CLN2 delivered to the brain. Shown from 5′ to 3′, the AAV vector encapsidates inverted terminal repeats (ITR) from AAV2 that surround an expression cassette consisting of the CAG promotor that includes the human cytomegalovirus immediate/early enhancer, a splice donor, the *left-hand* intron sequence from chicken β-actin, the *right-hand* intron sequence and splice acceptor from rabbit β-globin driving the expression of the human cDNA for CLN2 with an optimized Kozak translation initiation signal, and the polyadenylation/transcription stop from rabbit β-globin

terminal repeats, DNA packaging signal, and the replication origin. Replication and packaging of AAV vectors require all AAV and Ad helper functions to be provided in *trans*. AAV vectors are easily produced in sufficient yields compatible with clinical applications, and AAV vectors have been demonstrated to be effective in long-term gene transfer to the brain [17–24].

We have carried out two clinical studies for the treatment of the CNS manifestations of LINCL. We first used a gene transfer vector based on human serotype 2 (AAV2) and the second based on nonhuman primate serotype rh.10 (AAVrh.10). Both vectors use the same basic design, with the AAV2 or AAVrh.10 capsid, but the same expression cassette (Fig. 7.2).

At the time we initiated our program, there was extensive preclinical data with AAV in mouse models of metabolic disease, including lysosomal storage diseases, but there was no animal model of LINCL. Two human serotypes of AAV were available, AAV2 and AAV5 [25–30]. Of these two serotypes, as there was more experience with AAV2-mediated transgene delivery in the brain, we concluded that clinical development would progress more rapidly with AAV2 for the first generation study [31, 32].

For the development of our second generation study, we tested 25 serotypes of AAV (human and nonhuman) and identified 4 (AAV2, 5, 8 and rh.10) to be tested further for their ability to lead to widespread, persistent expression in the CNS [33]. These four serotypes were compared for their ability to transfer genes to the rat CNS. The data demonstrated that AAVrh.10 provided the most robust and widespread expression, including maximum spread beyond the site of administration

[34, 35]. It also mediated extensive axonal transport to distant cell bodies projecting from the sites of administration. Comparison of AAVrh.10 with AAV2 demonstrated that AAVrh.10 markedly improved outcomes in an LINCL knockout mouse model compared to AAV2 used in the first generation study [35, 36].

Independent of the choice of vector was the design of the expression cassette which regulates the magnitude, tropism, and persistence of the encoded protein. The expression cassette contains a promoter followed by the full length cDNA of the coding sequence of the transgene, CLN2. With the knowledge that current gene transfer technology only delivers genes to a limited number of cells in the CNS, the requirement to deliver the CLN2 protein product in a diffuse fashion suggested that high level constitutive expression was the most rational strategy to pursue. Assuming that there was no toxicology associated with constitutive production of TPP-I, the choice of a promoter was based on the available data regarding high level gene expression mediated by AAV2 vectors in the CNS [27, 36–38] (Fig. 7.2). An analysis of the published data for AAV2 at that time demonstrated that the chicken β-actin promoter with a CMV enhancer provided constitutive long-term expression in the CNS and has been used successfully in mouse models of lysosomal storage diseases [27, 36].

Delivery to the CNS

The brain is progressively atrophied in children with LINCL. Brain weight at autopsy is 500–700 g compared to the normal range of 1300–1400 g for children ages 6–10 years [3]. At the end stage of the disease, neuronal loss is so severe that it may be difficult to identify normal neuronal layers. A therapy that prevents or delays the disease progression would have a significant impact on quality of life and/or morbidity and provides a useful clinical study endpoint [14]. The CNS has the advantage of being at least partially immune-privileged, thereby reducing confounding effects of immune-mediated reduction in treatment outcome [39].

There are three potential routes of vector delivery to the CNS: intravascular, directly into the CSF via the intrathecal route or intracerebral (directly into the brain parenchyma; Fig. 7.3). Theoretically, intravascular delivery is the simplest strategy via the CNS arterial system. However, unless delivered under pressure (something not feasible in the CNS) or with disruption of the blood–brain barrier, AAV vectors do not penetrate CNS endothelium in amounts sufficient to treat LINCL.

Direct administration to the CSF has similar advantages to the intravascular route in that it is a relatively noninvasive procedure, and the CSF can be easily sampled for assessment of the efficiency of gene transfer. But the degree to which the ependymal cells at the brain/CSF interface will secrete enzyme through the brain parenchyma was unknown when we initiated clinical development for LINCL. Therefore, this route was not explored.

The most direct approach to administration of vectors to the CNS was administration to the parenchyma of the brain. However, there were challenges that required

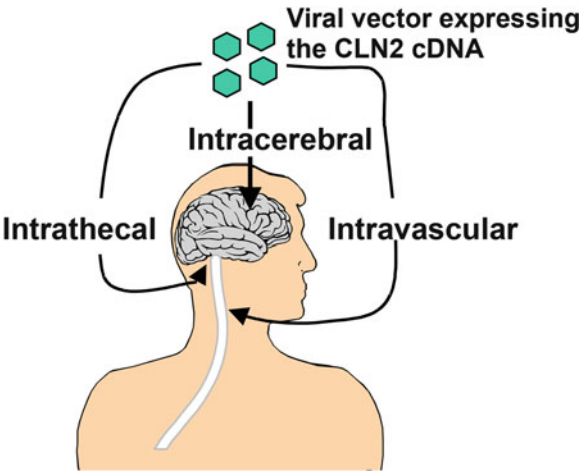

Fig. 7.3 Potential routes of vector administration. Theoretically, the AAV vector could be administered to the CNS via: (1) cerebral spinal fluid by the intrathecal route; (2) blood via intravascular administration; or (3) directly into the brain parenchyma via the intracerebral route

consideration. Direct administration to the CNS requires burr holes in the skull, and the requirement for diffuse administration likely necessitates administration into several sites. Without knowing the diffusion characteristics of the TPP-I protein in the CNS of a large experimental animal, it was not initially evident how many sites of administration would be required. The diffusion characteristics of the vector was an important consideration as administration of fluid (e.g., vector in the carrier vehicle) to the CNS has the limitation that the CNS is a closed space, thus limiting the volume that can be administered directly to the brain per unit time. Although we might have considered a reservoir to slowly administer the vector in a continuous fashion, this would have required studies of the reservoir/pump system regarding a number of issues, including safety, vector stability, and risk of infection and the use of an FDA approved device.

In summary, the delivery of the vector to the CNS was one of the major challenges in the development pathway. Based on the information available at the time, and balancing the numerous issues including safety and maximum vector dose, we concluded that the most feasible strategy for delivery to the CNS was simultaneous direct administration to multiple sites in the parenchyma.

Strategy for Preclinical Development

At the time that this project was first initiated, there was no LINCL experimental animal model available to test the proposed experimental treatment strategy. Based on the severity of the disease and lack of any available treatment, we decided that such a model was not critical to initiate the clinical study with a gene therapy for LINCL. Our rationale for proceeding with a clinical study was supported by two lines of evidence from experimental animal studies: (1) published studies by other

investigators regarding an experimental animal model of MPS VII, a lysosomal storage disease similar in characteristics related to LINCL [25, 27–29, 37] and (2) studies from our laboratory relating to the ability of an AAV2 vector coding for the normal CLN2 gene to deliver TPP-I to the CNS of normal animals [36].

Studies Regarding MPS VII LINCL and MPS VII have a number of similar features including the target organs which exhibit pathology. Each involves proteins which are normally secreted and taken up by neighboring cells utilizing the mannose 6-phosphate receptor pathway [14]. The consensus of the data on MPS VII mice was that local administration of AAV vectors to the CNS led to long-term expression of the therapeutic gene (β-glucuronidase) and that, over time, the spatial distribution of expressed protein increased [27]. The lysosomal storage defect was corrected over a wide area that greatly exceeded the area over which vector had spread and exceeded the obvious distribution of the enzyme detected histologically (cross correction). In addition, the clearance of storage defect lead to behavioral improvements in the AAV2 treated mice [25, 27–29, 37].

LINCL Preclinical Studies with AAV2 For LINCL, our experimental gene therapy studies in normal animals could be categorized into two steps. First, to develop methods to assess CLN2 expression and to show that the expressed TPP-I is functional, studies were carried out in vitro using plasmids and adenovirus vectors and in vivo using adenovirus vectors (Ad vectors were used because expression is far more rapid than that of AAV2 vectors, allowing fast turn around for the assessment of TPP-I detection methods). The data established that following gene transfer, TPP-I could be detected by enzymatic activity assay and immuno-based histological methods. In the absence of an LINCL animal model (at that time), all measures of efficacy were done with two independent surrogate markers for TPP-I detection in wild-type rats, an immuno-based method and the enzyme activity assay. We also demonstrated that viral gene transfer using an AAV2 vector could be used to locally produce enzymatic activity in excess of the expected 5 % of wild-type threshold deemed likely to be protective [5]. AAV2-mediated CLN2 delivery to the brain resulted in a robust expression of TPP-I that was primarily neuronal in nature, an observation that is consistent with the known tropism of AAV2 [3, 36]. Studies in experimental animals also demonstrated that delivery of the vector at multiple sites significantly increased the distribution of TPP-I, although distribution was somewhat limited by boundaries of the internal CNS structures. Importantly, expression of TPP-I was observed for at least 18 months (the longest time point evaluated) [36].

Strategy for Second Generation Preclinical Development

In parallel with AAV2 translation to clinic, we continued optimizing vector-mediated TPP-I delivery, facilitated with a newly available knockout LINCL mouse that closely resembled the human clinical disease phenotype [40]. As with all

product development, locking in a strategy is required to move forward, even in the context of continuous improvement at the laboratory level. Therefore, even as we proceeded with clinical development of an AAV2-based strategy, in the laboratory we sought the use of alternative serotypes for the development pipeline. We found that the AAVrh.10 serotype vector was the best vector to provide highest levels of TPP-I expression and could ameliorate LINCL disease phenotype in the LINCL knockout mouse, with improved survival [35]. An important finding was that near complete prevention of disease phenotype was observed with the earliest treatment, at 2 days of age, whereas treatment at 3 or 7 weeks had less impact on disease phenotype [41].

In order to translate these studies to a clinical setting, we also assessed the levels of AAV-mediated expression of TPP-I in a larger brain to evaluate the capacity of our delivery strategy and vector to effect widely distributed TPP-I protein. For this purpose, TPP-I levels were compared in nonhuman primates (NHP) that had been administered equivalent amounts of comparably produced AAV2hCLN2 or AAVrh.10hCLN2 vectors [42]. As a control, one nonhuman primate was injected with an equivalent volume of PBS. The vector (or PBS) was administered through catheters in each of 12 locations (bilateral through three burr holes) at two depths. Targeted locations were determined using MRI and CT scan imaging and were chosen to include areas of both white and gray matter. In total, eight rostral sites and four caudal sites were used. The animals were sacrificed at 90 days to assess distribution of TPP-I by peptidase enzyme activity. In the AAV2hCLN2 administered animals, 10 ± 6 % of the CNS had TPP-I levels >2 standard deviations above the background (assessed in the animal injected with PBS) while in the AAVrh.10hCLN2 administered animals, the coverage was 31 ± 8 % of the CNS, demonstrating the superiority of the AAVrh.10 vector [42]. Because the evaluation of the spread of the TPP-I protein was done in the context of 100 % normal endogenous TPP-I levels, the actual effective spread of TPP-I expression was likely much greater than that measured.

Translation to Clinic: Safety and Toxicology Studies

Once a surrogate measure of efficacy was demonstrated in rodents for the first generation AAV2-based study, a PreIND meeting was held with the Food and Drug Administration (FDA) in 2002. These discussions concluded with designs for the safety and toxicology studies that sought to mimic the anticipated clinical route and dose of administration with the clinical grade drug expressing the human CLN2 coding sequences. Both NHP (for the large brain) and rats (for large numbers) were used to evaluate effects of the transgene product and vector for the first generation vector [43]. Safety data from these studies was deemed acceptable for the clinical use of this vector and the transgene product. This data was leveraged for the second safety study which was initiated in 2006 with the AAVrh.10 vector, allowing significant reduction of the scope of the second safety study (less animals and shorter

duration postadministration) [42]. In the AAV2 nonhuman primate study, the route of administration evaluated was identical to that of the clinical study design and bracketed the weight adjusted dose [43]. For the AAVrh.10 nonhuman primate safety study, a single dose about tenfold higher than the clinical target was used in anticipation of an acceptable safety profile providing the basis for a clinical study design with dose escalation [42]. Importantly, while instillation of the vector has global implications for the large brain that must be evaluated, local impact to the site of injection does not scale with animal size and therefore requires careful histopathology evaluation. Weight adjusted, single dose studies with rats injected bilaterally into the striatum was used to evaluate safety at multiple time points [42, 43]. All of the standard safety-related parameters such as blood chemistry, hematology, and histopathology were assessed. Specific assessments for a surgical delivery of a gene therapy-based drug included immunity to the vector and animal behavior, with tissues reserved for biodistribution [36]. Germline transmission was not a concern, as the LINCL affected population does not reach childbearing age. The conclusion of each of the safety studies was that the vectors AAV2 and AAVrh.10 expressing the CLN2 gene had safety profiles amenable to clinical translation.

Regulatory Hurdles and Strategies

Clinical research with gene therapy has extensive regulatory oversight requiring a team with the expertise to understand the regulations, timelines, and reporting requirements. The coordination of compliance demands among the collective oversight groups at the national level (NIH, FDA, RAC) and the local level (IRB, IBC, DSMB) often have different and sometime mutually exclusive demands that have to be negotiated and in turn requires an iterative process that extends timelines to achieve fully informed authorities and their ultimate agreement. To manage this process, we have a regulatory team of trained personnel that have engaged both the investigators and the regulatory oversight bodies to negotiate changes through the regulatory maze and assure compliance.

The regulatory process to launch and conduct the clinical study was a multi-year timeline that started during the preclinical phase. The first step was to distill all the supporting data into a pre-IND (or a pre-Investigational New Drug) packet to be submitted to the FDA. This resulted in a consultation with the FDA, at which many questions relating to the path of translation to the clinical study were answered and guidance for future work provided. Ongoing discussions with the FDA leading up to the IND (Investigational New Drug application) followed the PreIND meeting. During the time between the PreIND and the IND, we designed the clinical protocol, which included discussions with a team of experts spanning many institutions and disciplines related to our study to provide guidance. The protocol was then submitted for review to the Institutional Review Board. After revisions it was submitted to the FDA as part of the IND and to the NIH Office of Biotechnology Activities-Recombinant DNA Advisory Committee as part of their submission and

review process. Lastly, the protocol was submitted to the Institutional Biosafety Committee and Data Safety and Monitoring Board. The study was initiated after being allowed to proceed from each of these oversight bodies with ongoing submissions throughout the course of the clinical study.

Clinical Strategy

Both of our clinical studies were designed to have two specific aims: (1) assess the hypothesis that direct administration of AAV-mediated delivery of CLN2 to the brain of children with LINCL could be achieved safely and with minimal toxicity and (2) within the constraint of a study design focused primarily on safety, to evaluate the hypothesis that direct administration of AAV-mediated CLN2 to the brain of children with LINCL would slow down or halt progression of the disease as assessed by an LINCL rating scale and quantitative MRI parameters [44]. Both studies were designed to have a follow-up time of 18 months post-vector administration.

Both studies were also designed not to be blinded. For ethical reasons, it is not possible to carry out a classic placebo controlled trial for gene therapy for the CNS manifestations of LINCL due to the invasive nature of the vector administration procedure [45]. Our best option was to design a study in which all families with the disorder fitting the inclusion/exclusion criteria were eligible, and children whose families elected not to participate were matched with children who did participate, and served as controls over time.

Both clinical trials were designed to assess direct CNS administration (12 locations in the brain) of AAV-CLN2 vector to children with LINCL. For the first study, following discussion with the FDA, children with moderate to severe disease received the vector. The decision to start with children with moderate to severe disease was made in order to maximize the risk/benefit ratio in the context of the experimental therapy and the invasive neurosurgery required for drug administration [44]. The surgery and related patient care is outside the scope of the chapter and has been published elsewhere [46]. During the time we conducted the first clinical study, a newly available knockout mouse model enabled the evaluation of the time of treatment in the course of disease progression and its impact on therapeutic outcome [40, 41]. The conclusion of these experiments was that the earlier the treatment the better the outcome suggesting that ultimate successful therapeutic intervention would require newborn screening and immediate follow-up with treatment [41]. Based on these results and the safety of the first study, the second clinical study was approved for the enrollment of children with early to moderate disease [47]. The treated children are being compared to 16 comparable untreated children. These children acted as “controls” and have been assessed by the same outcome parameters. All subjects are monitored before and after vector administration with a variety of safety measures and preliminary measures of efficacy [44].

For the gene transfer group, assessment is at screening, pre-transfer, 6, 12, and 18 months. For the screening/control group, this is at day 0 and 18 months. Because

the disease is rare and the children are from all over the world, it is our experience that the families of the control, untreated children will not participate if the children are required to be studied as often as at 6-month intervals. For this reason, and because the MRI procedure requires general anesthesia, we decided to have one follow-up time point for the control group, using 18 months to match the 18-month study period of the gene transfer group. This maximizes the chance of having the family return and minimizes the number of extra procedures done for the control study.

Safety Parameters

The safety issues regarding administration of gene transfer vectors are mostly generic and are similar to safety issues for the development of any new drug. Assessment of safety parameters was under strict standard operating procedures, with appropriate reporting under guidelines of the local IRB and IBC, the NIH OBA/RAC, and the FDA. A data safety monitoring board following appropriate guidelines (with specific stopping rules for the trial) assessed the safety reports from the trial. After an extensive pre-therapy evaluation that included baseline assessment of all safety parameters, the drug (vector) was administered followed by periodic assessment designed to determine if there were adverse events related to the administration procedure or the vector. These assessments were performed at set time points over the course of 18 months following vector administration and were superimposed on the general care of the patient. The safety parameters included: (1) general well-being (history, physical exam, with particular attention to neurologic exam); (2) hematologic, blood chemistry, and urine parameters; (3) EKG; (4) chest X ray; (5) specific neurologic parameters, e.g., disease specific rating scale, brain MRI, EEG; and (6) vector-specific parameters, including systemic host responses (anti-vector antibodies, cellular immunity against the vector). Some of these safety parameters (neurologic) were the same as those used for efficacy testing [44]. The outcome of the first study found no unexpected serious adverse events that were unequivocally attributable to the vector, although there were some serious adverse effects, the etiology of which could not be determined under the conditions of the experiment [47]. The second study is ongoing.

Efficacy Parameters

While there is excellent rationale for the development of gene therapy for the CNS manifestations of LINCL, proving that it is efficacious is a major challenge. For the first study, we used a clinical scale developed by Steinfeld et al. [48] with a modification that the vision parameter was excluded because we were not treating the eye and therefore did not expect to see treatment-related outcome. We did however

examine the eye as a function of disease progression for future studies [49]. MRI was also used as a quantitative parameter as a secondary outcome measure [50, 51]. In agreement with the FDA, we used established measures of function, including language, feeding, and ambulation, all directly connected to the disease.

At the start of the second trial, we had extensive natural history data from the ongoing screening study that provided the basis for a new clinical rating scale which enables the use of a new improved primary outcome measure for this study [52]. As an additional primary parameter, the screening study provided important MRI data that was used to establish a multiparametric correlate of disease progression and age. As secondary parameters, the Mullen scale [53, 54] and the Child Health Questionnaire [55, 56] are being used as exploratory quality of life measures.

In the first completed study, treated subjects as compared with untreated controls had a trend toward reduced rates of decline of all MRI parameters (a secondary parameter), although not statistically significant. The primary outcome variable (assessment of the neurologic rating scale) had a significantly reduced rate of decline in treated vs. control subjects. Although this trial was not randomized or blinded and lacked a placebo/sham control group, the primary outcome variable suggests a slowing of progression of LINCL in the treated children [47]. The second trial is ongoing with 8 of 16 subjects treated to date.

Assembling the Clinical Team

The conduct of a trial of the type described above requires expertise and time from a diverse ensemble of investigators including: neurologists and neurosurgeons, pediatricians, psychologists, anesthesiologists, ethicists, ophthalmologists, neuroradiologists, imaging experts, drug manufacturing, formulation and quality control personnel, pharmacists, and regulatory and clinical oversight personnel. The enlistment of this extensive collection of individuals, as well as the challenge for scheduling the team in the context of clinical resources and travel planning for the trial participants, makes this a challenge and requires a full time clinical coordinator.

Identifying Inclusion/Exclusion Criteria

In a small patient population such as those with LINCL, it is challenging to identify inclusion and exclusion criteria that enable a narrow range of phenotype free from confounding factors, yet broad enough to meet the scope of recruitment and yield statistically relevant results. In the case of LINCL, this was especially challenging. For the first study, the highly variable onset of clinical phenotypes and the paucity of genotype/phenotype correlations led us to use a less restrictive inclusion criteria. As a result of our screening study during the intervening years and the work of others, better correlates of disease progression with genotype were identified [52, 57, 58].

The second (AAVrh.10) study therefore was designed with a more restrictive set of inclusion criteria, which in turn limited the number of eligible participants. The second study benefited from an inclusion criteria for subjects with lower disease severity and thus an earlier age at enrollment which allows for the possibility of participation of younger siblings of affected children identified by genetic testing even in the absence of clinical phenotype.

Funding for Clinical Translation

Issues of funding are central to the initiation of most studies in translational research. A majority of clinical trials are supported by industry [59]. Unfortunately, a disease such as LINCL, which is an orphan disease, does not receive as much attention from industry because the potential for profit is low and the investment is high. Mounting of these clinical studies in an academic setting is financially challenging. This requires academic investigators to generate funding from disease-specific foundations and federal grants. Our first study was partially funded by a family foundation and our second study partially funded by a federal grant.

Lessons Learned

After more than a decade and a half of executing the translation of the therapeutic concept for treating LINCL, we have overcome numerous hurdles with each informing and improving the process. Many lessons relate to the hard science of drug design, manufacture, clinical study and conduct, but numerous challenges are related to the relationship between researchers, patient families, and funding agencies.

For the trial execution, nearly all of the challenges relate to the participant; either coordinating the travel, follow-up, or evaluation of the outcome. Because the disease is rare and affects individuals across the globe, we found that screening and post-treatment follow-up was a challenge, in particular for families with children that had very special needs in transportation and housing. As a result, we began to provide remote follow-up via video conference call in conjunction with the patient's local medical team or sending our clinical staff, including a physician to the participant's home base. This dramatically improved participant compliance. In addition, since the rating scale has subjective parameters, we have devised improvements to maximize the integrity of the data. For example, the assessment of the clinical rating scale (the primary outcome) is evaluated by a study neurologist and this is videotaped. The video is then reviewed and scored by three additional neurologists who are blinded to the participant's treatment status. The final score is a composite of the four reviewers, minimizing bias and subjective interpretation. To transition to a more objective outcome, we have identified numerous MRI parameters that characterize the CNS for correlates with disease progression, an example being the simple

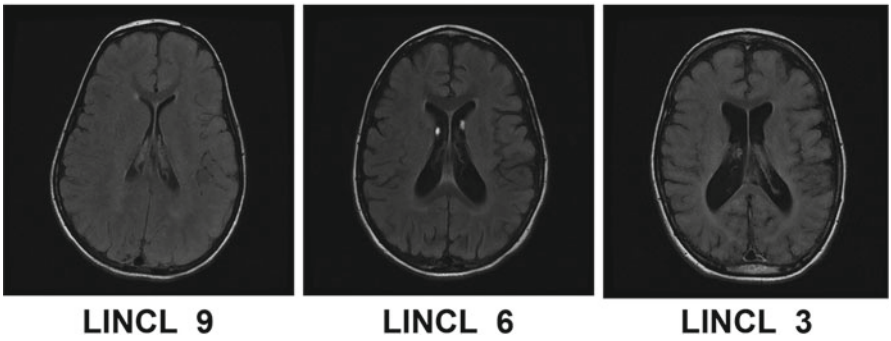

Fig. 7.4 Examples of MRI images at different stages of LINCL disease progression. The MRI demonstrates increasing volumes for the CSF filled ventricles and decreasing brain gray matter as disease progresses from the score of 9 to 3 on the Weill Cornell LINCL rating scale [52]

measure of gray matter/ventricular volume provided here (Fig. 7.4). Even with the most comprehensive review of the participant's abilities and behavior, there is variability in these measures. Our studies to date suggest that MRI can provide a better scale for disease progression [51].

The second category of challenges relate to the families of the participants. These issues are complex and requires cooperation of ethicists and the clinical team addressing issues and possible solutions [45, 60, 61]. Probably, the biggest issue in a trial for a fatal disease with no alternative options is the fact that an informed consent can state that the treatment is unlikely to be efficacious but parents are more likely to disregard any such statement since there is no alternative. Along these lines, there is an entire spectrum of emotions from parental guilt for the inherited fatal disorder, through a misunderstanding of the intent of early phase clinical trials and expectations of risk/benefit, all of which must be dealt with in a rational and compassionate way with documented acceptance of informed consent. The conduct of these CNS gene therapy studies is further complicated in the context that sources of funding for orphan disease clinical trials often come from individuals with a personal investment in finding a treatment for the disorder. This raises the issues relating to competing interests between funders and investigators and the need to safeguard against these conflicts of interest. We encountered these issues during the course of our studies and established and implemented safeguards in conjunction with our ethicist co-investigators [60].

Summary

Here, we have described our development of a therapeutic for an inborn error of metabolism. We have presented each step from therapeutic concept through clinical trial and provided a road map that we hope will inform others who seek to do the

same in an academic setting. Described are the hurdles, anticipated and not, which had to be overcome for the clinical study to be viable and informative. Importantly, the many lessons learned with a first generation drug and clinical trial should be used to improve future generation products or studies which can and should be developed concurrently.

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Chapter 8
Gene Therapy for Spinal Cord Injury

Ioana Goganau and Armin Blesch

Abstract New insights into the pathophysiological mechanisms that contribute to the limited functional recovery after spinal cord injury (SCI) have led to the development of several novel experimental approaches. Gene therapy is one means to identify and modulate molecular mechanisms that promote plasticity and regeneration in the injured spinal cord. Therapeutic targets that can be pursued by gene delivery include the intrinsic regenerative capacity of injured neurons, the rearrangement of spared circuitry, and extrinsic factors in and around the lesion site limiting axonal regeneration. An increasing number of animal studies have also employed gene therapy in combinatorial treatments to tackle the complexity of injury-induced changes. However, several challenges regarding the efficiency, safety, and regulation of gene expression remain to be addressed. This chapter aims to summarize different strategies for gene transfer after SCI and their translational potential.

Keywords Regeneration • Plasticity • Combinatorial treatments • Functional recovery • Cell transplantation • Viral vectors • AAV • Lentivirus • Retrovirus • Regulated gene expression

Introduction

Spinal cord injury (SCI) leads to the interruption of axonal pathways and the loss of neurons and glia often resulting in persistent sensory, motor, and autonomic deficits. Similar to severe injuries in other parts of the central nervous system (CNS), spontaneous recovery is limited, resulting in serious lifelong impairment. Even partial injuries lead to fundamental alterations in the structure and function of remaining neuronal connections. While some mechanisms and potential therapeutic approaches are common to SCI and other CNS injuries, the unique functional role and anatomy

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of the spinal cord pose specific challenges. These include the close proximity and local connectivity of motor and sensory systems and the interaction of the local circuitry with long descending and ascending projections. The risk for adverse effects like pain and spasticity from broadly acting interventions are therefore potentially higher than in other CNS structures. Targeted gene delivery might be one means to limit effects spatially and temporally, and thereby restrict plasticity and regeneration to specific neuronal populations after SCI.

In contrast to progressive changes in neurodegenerative disorders requiring long-term therapeutic interventions, the expression of therapeutic genes after SCI might become undesirable once beneficial effects have been established, to avoid adverse effects or to allow for new connections to function properly. Despite these challenges, experimental gene delivery has led to important insights into mechanisms impeding functional recovery after SCI and the development of means to improve functional outcomes. Factors addressed by gene therapy include cellular and biochemical barriers to spinal cord regeneration extrinsic to injured neurons and their axons such as growth-inhibitory molecules, inflammation, gliosis, and cystic degeneration. In addition, gene delivery can provide guidance cues that are absent in the adult CNS, stimulate axonal growth towards appropriate targets [1], and induce transcriptional programs that are insufficiently upregulated in injured neurons, but vital for regeneration [2]. Thereby, multiple interacting mechanisms can be modulated to activate regenerative programs that are also key to regeneration in the peripheral nervous system (PNS) [3] and axonal growth during early development [4]. By combining gene transfer with other interventions, synergistic effects on functional recovery can be achieved.

In this chapter, we aim to summarize recent data on gene therapy for spinal cord regeneration, sprouting and plasticity, strategies for localized gene delivery, and the challenges in clinical translation.

Gene Delivery for Axonal Growth Using Ex Vivo Modified Cells

Cell transplantation is one of the most extensively studied approaches to enhance axon regeneration and to reconstitute the spinal cord parenchyma in animal models of SCI. Besides transplantation of peripheral nerves, stem cells, fetal tissue, and naïve cells, grafting of cells genetically modified to express potential therapeutic genes (ex vivo gene therapy), either alone or in combination with other interventions, has been investigated extensively over the last 25 years (Fig. 8.1). Advantages of ex vivo gene delivery include the possibility to comprehensively characterize cells and gene expression in vitro and the ability to restrict gene expression in vivo to grafted cells. Thereby, depending on the migratory potential of cells, the biodistribution of gene products can be spatially restricted to a graft site, while preventing potentially detrimental effects in other parts of the nervous system.

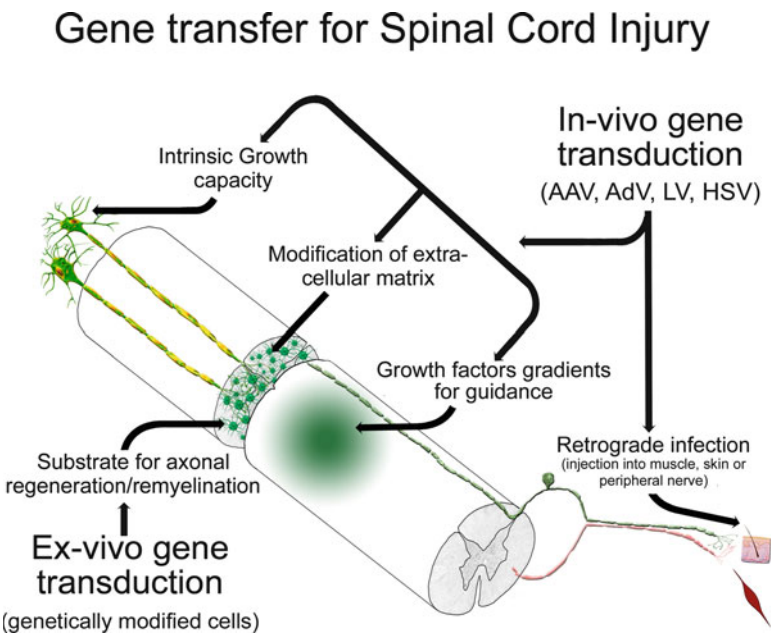

Fig. 8.1 Schematic illustration of gene therapy for axonal regeneration. In vivo gene transfer via adeno-associated virus (AAV), lentivirus (LV), adenovirus (AdV), or herpes simplex virus (HSV) can transfect neurons and/or glia in the spinal cord by direct intraparenchymal injection or by retrograde transport from muscle, skin, or peripheral nerve. Genetically modified cells (ex vivo gene transfer) can be grafted to the lesion site to provide a substrate for axonal growth or to remyelinate spared axons

The implantation of cells embedded in collagen or fibrin matrices can further restrict the distribution of cells and gene expression to an area of interest. In addition, transplanted cells expressing a gene of interest may also serve as a substrate for axonal growth, fill cystic cavities, or remyelinate axons, thereby modifying the lesion environment, which is inhospitable to axonal regeneration.

Ex vivo gene delivery can be accomplished by viral transduction of cells, most commonly using retroviral vectors. In addition to homogeneous, genetically modified cell populations such as fibroblasts [5–19], bone marrow stromal cells [20–22], Schwann cells [23–27], and olfactory ensheathing cells [28–30], more heterogenous grafts including neural stem cells [31–34] and organotypic transplants such as predegenerated peripheral nerves [35, 36] have been transplanted to the injured spinal cord after in vitro genetic modification.

Neurotrophic factors including the neurotrophin family comprise one class of genes that has been the subject of numerous ex vivo gene therapy studies in animal models of SCI. Since the discovery of nerve growth factor (NGF) over 60 years ago [37], it has become clear that neurotrophins not only influence neuronal survival, axonal growth, and target innervation during development but also in the adult PNS and CNS.

The systemic administration of growth factors is complicated by the inability of these large polar molecules to cross the blood brain barrier and broad adverse effects on non-targeted structures. In contrast, delivery by genetically modified cells is an efficient means for long-term, stable, localized intraparenchymal expression in the injured spinal cord. Once grafted into a lesion cavity, either as cell suspensions or embedded into an extracellular matrix, cells can serve as biological minipumps expressing neurotrophic factors for extended time periods in vivo. In addition to members of the neurotrophin family including NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) NT-4, and a NT-3-derived multi-neurotrophin named D15A [38], other growth factors such as neuropoetic cytokines [leukemia inhibitor factors (LIF), interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF)], glial cell line-derived neurotrophic factor (GDNF) family ligands, and insulin-like growth factors (IGF) have been investigated for their axon growth-promoting properties using cellular delivery.

Similar to findings using intraparenchymal infusion of trophic factors, sensory axons tend to show the most robust growth responses compared to other neuronal populations after cellular growth factor delivery. Specifically, dorsal column sensory axons have been shown to extend into NT-3-, BDNF-, and GDNF-expressing grafts [17, 20, 21], whereas peptidergic and non-peptidergic nociceptive axons respond to NGF and GDNF, respectively [17, 39]. Growth of reticulospinal, raphaespinal, rubrospinal, and propriospinal axons can be augmented by cellular expression of BDNF and NT-4/5 [8, 9, 11, 14, 18, 20, 23]. Because propriospinal neurons may serve as relays for transmitting supraspinal signals from injured axons to neurons below the lesion [40], responses of this class of neurons to cellular growth factor delivery are of particular interest. Besides BDNF and NT-4/5, GDNF-expressing fibroblasts [17] and Schwann cells [41] have also been shown to increase propriospinal axon growth.

While growth factor delivery by cells grafted into the lesion site increases the number of regenerating axons from a wide range of spinal projection neurons, axons usually only extend into but not beyond a lesion site. Continued high levels of growth factors in the lesion, the lack of an axon growth-promoting stimulus beyond the lesion site, and inhibitory molecules in the surrounding host parenchyma contribute to the lack of sustained axon growth. Combinatory approaches are needed to achieve bridging axon regeneration in conjunction with cellular growth factor delivery.

Given the importance of the corticospinal tract (CST) in fine motor control in human and nonhuman primates, neurotrophic factor gene delivery to enhance CST regeneration and to protect pyramidal neurons from cellular atrophy is highly relevant. NT-3, one of the first neurotrophic factors to demonstrate functional improvements in animal models of SCI [42], enhances short distance sprouting of CST axons in the remaining host gray matter when expressed by retrovirus transduced fibroblasts or adenovirus (AdV) transduced peripheral nerves or olfactory ensheathing cells that are grafted to a spinal cord lesion [15, 29, 36, 43]. However, CST axons do not elongate for long distances around or significantly into a lesion site. Indeed, the quest for a favorable matrix for CST axon regeneration continues, and only fetal transplants seem to provide a substrate suitable for CST axon penetration either

alone or in combination with infusions of NT-3 [44]. Interestingly, lesion-induced atrophy of CST neurons cannot be prevented by cellular NT-3 delivery to the spinal cord in rodents and primates despite its axon growth-promoting effects [19]. To prevent axotomy-induced corticospinal neuronal atrophy and cell death, the only neurotrophin shown to be effective is BDNF delivered by a cellular graft in the spinal cord or infused into the cortex [19, 45]. Curiously, BDNF or NT-4/5 binding to the same tyrosine receptor kinase (trkB) does not promote CST growth when provided by genetically modified fibroblasts or bone marrow stromal cells at the lesion site [12, 18, 19], though it does stimulate sprouting when it is applied at the cortex [46]. These differences might be due to the distribution and the amount of receptors or different signaling in axon and cell soma. This hypothesis is supported by studies overexpressing the BDNF receptor trkB in adult layer V corticospinal neurons, together with subcortical BDNF-expressing cellular grafts [47]. Only with this combination, and BDNF sources close to the cell soma, CST axons enter BDNF expressing grafts. Other growth factors delivered by genetically modified cells at the lesion site, such as IGF-1, also fail to promote CST growth in the adult injured spinal cord, despite the importance of IGF-I for corticospinal neuron development [48, 49].

In contrast to CST neurons, brainstem-derived projections such as rubrospinal neurons show axon growth responses to BDNF, whether it is infused [50, 51] or expressed by lentiviral or AAV-mediated gene transfer [52, 53] at the level of the cell soma in the midbrain. Growth of rubrospinal axons is also enhanced when BDNF is expressed at the lesion site by fibroblasts [11] or olfactory ensheathing cells [28].

While most studies have investigated effects of growth factor secreting cells grafted immediately post-injury, studies indicate that axons remain responsive to neurotrophic factor gene delivery even at extended, chronic injury time points, when the development of a glial scar, retraction of axons from the lesion site, and neuronal atrophy pose additional challenges for axon regeneration. Indeed, grafts of NT-3 and BDNF-expressing cells allow axons to cross the glial scar and overcome the inhibitory extracellular matrix around the lesion site [10, 22], tipping the balance between inhibition and stimulation of axonal growth. Overall, similar although sometimes less pronounced effects on axon growth are observed in chronic SCI sometimes with modest functional recovery [8, 9, 13, 14, 16, 43].

Cellular neurotrophic factor gene delivery has also been combined with other interventions including in vivo gene transfer, modifications of the extracellular environment, or activation of neuron-intrinsic gene programs for regeneration (see below).

In Vivo Gene Delivery to the Spinal Cord

The studies described above clearly show that cellular gene transfer can promote sprouting and/or regeneration in the injured spinal cord. However, genes that need to be expressed in injured host neurons or other host cells require strategies for

direct in vivo gene delivery. Viral vectors for efficient and safe gene transfer have become widely available in recent years and together with appropriate targeting and molecular switches, virtually any area of the CNS can now be genetically modified. The efficacy of viral gene transfer is dependent on virus and host characteristics including virus type, serotype/variant, vector design, retrograde transport efficiency, place of injection, and presence of cellular receptors. Vectors can be administered in the vicinity of lesioned axons in the spinal cord, to the cell body of targeted neurons such as dorsal root ganglia (DRGs), red nucleus or cortex, or in the cerebrospinal fluid via intrathecal injections, as well as to the skin, muscle, or peripheral nerve for axonal uptake and retrograde transport (Fig. 8.1).

Lentivirus (LV) and AdV-based vectors have a pantropic nature, transducing neurons and other cells such as astrocytes or ependymal cells after injections in the spinal cord [54, 55], while adeno-associated virus (AAV) and herpes simplex virus (HSV) are primarily neurotropic [56, 57]. The specificity of gene expression can be further restricted by cell-specific promoters.

LV injections in the spinal cord result in stable transgene expression for months to years, with minimal inflammatory response and tissue damage [54, 58, 59], making the system suitable for long-term delivery of therapeutic molecules, if specificity for a certain cellular population is not required (Fig. 8.2). Virus spread in the spinal cord can be varied by injection volume, virus titer, and speed. Spread along white matter tracks is more pronounced compared to gray matter injections, in the range of 3–4 mm from the site of the injection [21, 54]. Like all integrating vectors, LV poses a risk of insertional mutagenesis. Non-integrating/integration-deficient LV might decrease this risk while maintaining rather stable expression in nondividing cells [60]. Indeed, integration-deficient LV seems to efficiently target motor neurons and interneurons after spinal cord injections [61]. However, there is evidence of lower gene expression and a decline in gene expression over time especially in dividing cells with non-integrating LV [61–63].

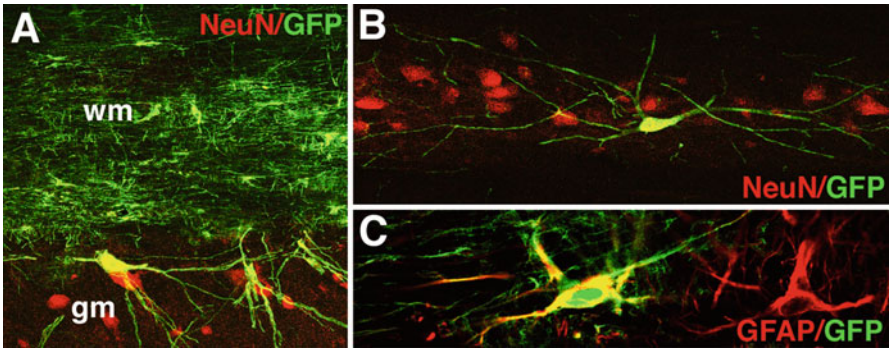

Fig. 8.2 Lentiviral gene transfer in the adult spinal cord. Vectors expressing the reporter gene GFP (*green*) can infect (**a**, **b**) neurons (NeuN label, *red*) and (**c**) glia (GFAP label, *red*) 6 weeks post-injection. *gm* gray matter, *wm* white matter)

Replication-defective AdV can effectively transduce neurons in the spinal cord as well as astrocytes and other infiltrating cells in the lesion site [55]. The level of expression is mostly dose-dependent [54], but gene expression is rapidly downregulated, partially due to the loss of vector in dividing cells and partially due to viral toxicity and inflammation. In the spinal cord, expression can be detected up to 3 weeks [55, 64], making AdVs potentially interesting when short-term gene expression is desired. However neuronal and glial degeneration and death caused by inflammatory reactions in response to adenoviral coat proteins [65] limit the usefulness of AdV.

HSV has also been investigated for use in several CNS pathologies [56] and represents another vector for spinal cord gene transfer, although less frequently used. Its strong neuronal tropism and its efficient retrograde transport after peripheral injections make it an interesting gene therapy vehicle [56, 66]. Several modifications have been made to ensure safety, and current amplicon-based HSV-1- vectors are replication-deficient, maintaining only a small fragment of the original genome and allow for independently regulated expression cassettes [67].

One of the most promising and well-characterized vector systems is AAV, due to the lack of any pathology in humans, lack of viral gene expression, and a high affinity for neurons. The infectivity of AAV in the CNS depends primarily on the dose and serotype that is used [68–71]. At least 12 different serotypes (AAV1–12) and more than 100 variants with different properties have been described [72, 73]. Thus, AAV is very versatile, and depending on the cellular target, an appropriate serotype can be chosen. For example, AAV 1, 5, and 6 are most efficient for transduction of DRGs neurons [70], while AAV 1, 4, and 6 seem to be most efficient for transduction of motor neurons [74, 75], AAV1 for corticospinal neurons [76] and AAVrh10 seems to infect not only neurons but also spinal glia [77].

Retrograde infection of the spinal cord by peripheral injections into skin, muscle, or peripheral nerve would further limit the invasivness of virus administration and enhance the translational potential. Different viral systems including AdV, HSV, lentivirus, and AAV can to some extent be taken up by axon terminals in the periphery and transported to motor neurons in the spinal cord or sensory neurons in the DRGs [64, 66, 74, 78–85]. However, the majority of peripherally injected viral particles remain in the vicinity of the peripheral injection site. Modifications in the viral coat have been one approach to enhance retrograde transport. Lentivirus, pseudotyped with rabies virus glycoprotein, shows enhanced retrograde axonal transport after peripheral injection compared to the more commonly used vesicular stomatitis virus G-protein (VSV-G) envelope [85]. The efficiency of AAV in retrograde transduction is also influenced by serotype. After injection into the sciatic nerve or muscle, AAV1 is superior to AAV2, 3, 4, 5, or 6 in retrograde transduction of motor neurons [74]. Retrograde transport of AAV and AdV after injection in peripheral nerves is also improved by prior demyelination of peripheral nerves [82, 86]. Another strategy to enhance retrograde transport is the use of a genetically engineered double-stranded variant of AAV [self-complementary AAV (scAAV)] [87, 88] compared to the wild-type single-stranded AAV (ssAAV). Because the synthesis of the second strand is a rate-limiting step in viral infection, the apparently

enhanced retrograde transport of scAAV might be more related to increased infectivity than retrograde transport. While the available space for gene expression cassettes in scAAV is reduced by 50 %, scAAV is about 20 times more effective in retrograde motor neuron infection after intramuscular injection than ssAAV [74]. scAAV variants are also superior to ssAAV when administered intrathecally with a high specificity for DRG neurons. scAAV1 appears to be superior to scAAV5 in transducing DRG neurons after intrathecal injection [89], and even higher infectivity has been reported for AAV8 [90]. Modifications of the AAV capsid by directed targeted evolution has also shown promise by increasing cell tropism and retrograde transport [73, 91] and might further improve the efficacy of retrograde AAV gene transfer.

Temporal Regulation of Gene Expression

The majority of studies using ex vivo and in vivo gene transfer have used constitutively active promoters resulting in persistent, long-term gene expression although decreases in amount of gene product over time have been reported depending on the type of vector used [54, 68]. An external control over gene expression using regulatable promoters or promoters that automatically shut off would provide a means to control the amount and duration of gene product. Indeed, permanent or high expression of genes that modulate regeneration and plasticity can have long-term deleterious effects. Long-term delivery of neurotrophic factors such as NT-4 and GDNF can lead to an increase in graft size [17, 18]. High levels of continuous BDNF delivery by AAV have also been shown to increase the excitability of spinal neurons leading not only to enhanced axon growth but also to spasticity [92, 93].

Besides improving the safety of gene delivery, regulated gene delivery might also be needed for long distance axon regeneration. The continuous supply of growth factors within a spinal cord lesion site and a lack of growth-promoting stimuli beyond a lesion may prevent bridging axon regeneration across an injury. Gradients of growth factors in the developing CNS and PNS that change over time and contribute to topographic target innervation by growing axons may be equally important for regenerating axons [94–98].

Studies in the injured rat spinal cord have shown that the transient doxycycline-regulated expression of BDNF by genetically modified fibroblasts is sufficient to sustain axons that have regenerated into a spinal cord lesion site [99]. However, turning off growth factor gene expression in the lesion site is insufficient for axons to extend into the distal spinal cord. Glial scar, inhibitory extracellular matrix, and a lack of additional growth stimuli beyond the lesion site likely contribute to the lack of continued growth. In contrast to BDNF-responsive axons in the lesion site, the density of NT-3-responsive dorsal column sensory axons that have regenerated into and beyond a lesion after regulated lentiviral NT-3 expression declines, once NT-3 gene expression is turned off [100]. A loss of axon collaterals with declining

NT-3 expression and a lack of functional synapses within and beyond the lesion site might contribute to the reduction in the density of regenerated axons. Indeed, axons that are sustained after NT-3 expression is turned off form axoglial synapse-like structures. Thus, similar to axon pruning in development, sustaining regenerated axons might depend on the formation of active synapses.

In addition to promoters regulated by the administration of doxycycline, virus known to turn off gene expression after a defined time frame such as HSV and promoters that shut down with a changing environment or with the differentiation of cells expressing a transgene can be used for transient gene expression. Examples for these approaches include the use of hypoxia-sensitive promoters [101, 102], primarily active shortly after injury, or a nestin promoter, which is turned off once grafted neural precursor cells differentiate [103]. Other approaches to temporally stimulate growth factor signaling in injured neurons and axons include the expression of modified neurotrophin receptors with regulated kinase activity, that can be activated by small synthetic ligands or light, restricting the downstream receptor signaling to the time period of ligand- or light-mediated activation [104, 105].

Taken together, regulated gene expression would not only clearly improve the safety of gene delivery by discontinuing gene expression in case of adverse events but also allow for appropriate dosing and timing of gene expression to maximize functional benefits.

Directional Growth of Axons and Target Innervation

In vivo and in vitro studies investigating peripheral nerve regeneration have shown that chemotropic guidance by regulated neurotrophic factor expression induces directional axonal elongation of the growth cone [106, 107]. Schwann cells upregulate NGF, BDNF, GDNF, CNTF, and other trophic factors in particular in the distal nerve stump, resulting in chemotropic growth factor gradients. Upon target reinnervation, when growth factors are no longer needed, expression decreases [108–112]. Thus, axons are guided by a regulated spatial and temporal expression pattern [113–116]. Although neurotropic guidance does not occur spontaneously in the adult injured CNS, injured CNS axons seem to be responsive to growth factor gradients generated by in vivo gene transfer within the adult CNS. A gradient of NT-3 distal to a lesion site not only increases dorsal column sensory axon growth, but allows for axons to bridge for short distances across a cervical lesion site [21] (Fig. 8.3). Expressing NT-3 in nucleus gracilis, the target region of hindlimb dorsal column sensory axons can be used to guide axons to their original target, where axons form appropriate synapses [117]. Similarly, descending reticulospinal fibers extend beyond a lesion site filled with bone marrow stromal cells only when BDNF is also expressed in the distal spinal cord [93]. Axons from grafted neural restricted precursors also sprout towards the highest BDNF concentration [118], and when a lentiviral BDNF source is located in the medullary dorsal column nuclei, target innervation

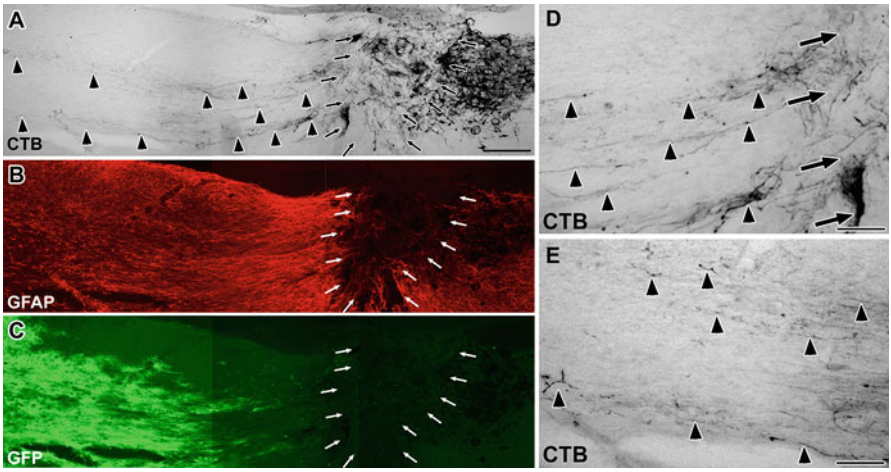

Fig. 8.3 Axonal bridging across a cervical dorsal column lesion in animals that received a combination of bone marrow stromal cell grafts at the lesion site, a chemotropic source of NT-3 lentivirus rostral to the lesion site and a conditioning lesion to activate the intrinsic growth capacity of sensory neurons. (**a**) CTB labeled axons extend across the lesion site (*arrows*) filled with bone marrow stromal cells. Axons (*arrowheads*) extend into the host tissue identified by (**b**) GFAP labeling towards lentiviral NT-3 expression identified by (**c**) GFP. (**d**, **e**) Higher magnification of CTB labeled axons at (**d**) the rostral host/graft interface and (**e**) in the rostral spinal cord. Rostral is to the *left*. Scale bar = 200 μm in (**a**–**c**), 75 μm in (**d**, **e**)

and synapse formation can be achieved [119]. The same directed growth has also been shown for DRG neurons transplanted in the corpus callosum that extend axons towards a viral NGF source [120] or across the dorsal root entry zone towards areas of highest neurotrophin expression [121]. These responses can be further refined by chemorepulsive guidance cues such as semaphorins, thereby restricting the reinnervation pattern of sensory axons in the spinal cord [122] or increasing the number of DRG axons turning away from a repulsive guidance post of semaphoring-expressing cells [123].

Besides chemotropic guidance, physical guidance of axons by linear scaffolds can support a directional, linear growth pattern [124–126]. In combination with genetically modified cells and distal growth factor gradients, axons elongate in a linear fashion within this scaffold throughout an extended lesion site [127, 128]. This is of particular importance when translating findings to the injured human spinal cord, due to the larger distances that need to be covered compared to animal models.

Taken together, these studies show that gene therapy in combination with other interventions could be successfully used to provide attractive and repulsive cues in a regulated spatio-temporal manner to direct the growth of regenerating adult axons after SCI.

Activating the Intrinsic Regenerative Program by Gene Delivery

The failure of CNS neurons to upregulate regenerative genetic programs is one major factor contributing to the limited axonal regeneration after SCI. An inadequate expression of proteins necessary for axon growth [129, 130], a lack of intracellular signal activation [131], and insufficient protein synthesis [132] can lead to cellular atrophy [51] or neuronal death [45]. In contrast, lesions in the PNS lead to substantial, long-term changes in the expression of thousands of genes in sensory neurons until target innervation is achieved [3, 133, 134]. Injury in the peripheral axon segment of DRG neurons results in a so-called conditioning lesion [135, 136], characterized by transcriptional changes that contribute to an enhanced intrinsic regenerative capacity [2] even after a spinal cord lesion [136, 137]. These findings indicate that an appropriate genetic program might be a prerequisite for long-distance axonal regeneration, and when combined with other methods addressing factors in the environment of injured axons, could synergistically promote axonal growth. Because this topic is discussed in a separate chapter in this book (Zhigang He), we will focus on studies employing gene therapy or a combination of gene therapy with the activation of the intrinsic regenerative capacity.

Several studies have shown that activation of gene programs by conditioning lesions can increase the number and distance of sensory axons bridging across a lesion site filled with BMSCs towards neurotrophin gradients established by lentiviral gene transfer [117, 134, 137]. Importantly, regeneration is even enhanced, when peripheral neurons are conditioned after the spinal cord lesion in a subacute or chronic model of SCI, suggesting that activation of gene programs will also be effective in chronic SCI and is therefore clinically relevant [134, 137]. The mechanisms underlying this intrinsic growth program and replacing conditioning lesions with more practical means are of particular interest. However, the underlying signaling cascades remain incompletely understood, and several signaling pathways are likely involved. These include increases in cAMP levels [138, 139] and activation of CREB (cAMP response element binding protein)-dependent transcription [140, 141], upregulation of activating transcription factor 3 (ATF3) [142, 143], activating signal transducer, and activator of transcription-3 (STAT3) and SMAD signaling via neuropoetic cytokines (IL-6/LIF/CNTF) [144–147] and bone morphogenic proteins [148]. Compared to conditioning lesions, pro-regenerative effects of each single factor are smaller, suggesting that synchronous initiation of several transcriptional programs is required to fully activate signaling cascades for axonal regeneration.

Another direction to stimulate the intrinsic growth capacity is the overexpression or downregulation of factors important in CNS development that are down- and upregulated, respectively, in adult neurons to reconstitute the intrinsic conditions of younger neurons. For example, lentiviral overexpression of the transcription factor retinoic acid receptor beta (RARß2) enables the regeneration of adult DRGs across the inhibitory dorsal root entry zone [149], as well as growth of descending CST

fibers after a mid-cervical lesion [150]. CST regeneration is also enhanced by expressing neuronal calcium sensor-1 (NCS-1) resulting in increases PI3K/Akt signaling [151] in pyramidal motor neurons. In CST neurons, downregulation of mammalian target of rapamycin (mTOR) activity, by its negative regulator PTEN (phosphatase and tensin homolog), appears to contribute to a lack of regeneration after injury [152]. In vivo downregulation of PTEN via intracortical injections of shRNA-expressing AAV enhances CST regeneration/sprouting into and around the lesion and some motor recovery [153]. Moreover, concurrent activation of mTOR and STAT3 pathways via CNTF/LIF, achieved by double deletion of PTEN gene and Suppressor of Cytokine Signaling-3 (SOCS3), leads to greatly enhanced and sustained regeneration in the optic nerve [154]. Taken together, these studies indicate that general principles in the activation of intrinsic regenerative programs exist, and that such strategies could be further developed to support regeneration in the injured spinal cord.

Nonneuronal Targets for SCI Gene Therapy

In addition to direct effects on injured neurons or axons, genetically modulating the cellular and extracellular environment may also result in functional benefits. Indirect targets include inflammatory processes, the cellular composition at the lesion site, and inhibitory components of the extracellular matrix.

Inflammatory processes including the recruitment and activation of glia and inflammatory cells, and the release of cytokines, proteases, and growth factors after SCI contribute to a dynamic lesion environment and can lead to excitotoxicity and apoptosis of neurons and glia [155]. Anti-inflammatory pharmacological approaches with IL-10 indicate improved functional outcomes in animal models of SCI [156]. Using HSV-mediated gene therapy, transient IL-10 expression also seems to downregulate pro-inflammatory molecules, thereby increasing neuronal survival [157] and decreasing below-level pain after SCI [158], findings that are similar to previous studies with AdV [159].

Manipulations of the inhibitory environment in the injured spinal cord including digestion of chondroitin sulfate proteoglycans (CSPGs) and the neutralization of myelin-based inhibitors (Nogo, MAG, OMGP) and other repulsive molecules [160, 161] have mainly been approached pharmacologically. To date, only a limited number of studies have directly manipulated extracellular matrix, cell adhesion, and astroglia by viral gene transfer. More frequently, digestion of CSPGs by enzyme delivery was combined with cellular or viral delivery of neurotrophic factors. In general, growth responses to cellular and viral neurotrophic factor delivery are enhanced by digestion of CSPGs. For example, digestion of CSPGs by chondroitinase ABC can increase sensory axon growth into brainstem target nuclei in response to lentiviral NT-3 gene transfer up to 10-fold [162] or modulate spinal cord plasticity in conjunction with AAV-NT-3 [163]. Only recently, lentiviral and cellular ChABC delivery has been investigated as a means for long-term efficient CSPG

digestion. These studies indicate that lentiviral ChABC is more efficient than enzyme administration [164, 165] leading to increased tissue sparing and functional improvement after SCI [164]. In addition, Schwann cells expressing ChABC in combination with the multi-neurotrophin D15A can increase the number of propriospinal and brainstem axons beyond a contusion lesion [26].

Not surprisingly, modifications of the response to cell adhesion molecules (CAMs), for example, by modifying CAMs by delivery of polysialyltransferase (PST) [166] or by expressing receptors that interact with the extracellular matrix, such as alpha9beta1 integrin [167], increase axon growth.

Modifying the cellular composition of the lesion site by manipulating infiltrating cells might also be an interesting therapeutic target that can be addressed by gene therapy. Astrocytes contributing to the inhibitory scar around the lesion react to overexpression of TGFα after AAV injections with enhanced migration, proliferation, and filling of the lesion site facilitating axonal penetration into and potentially beyond the injury site [168]. Ongoing studies converting glia into neurons by lentiviral sox2 expression offers new prospects for the activation of intrinsic stem cells by gene therapy [169]. Tissue sparing and angiogenesis at a spinal cord lesion can also be stimulated by increased vascular endothelial growth factor (VEGF) expression via AAV and AdV delivery of an engineered transcription factor to induce VEGF transcription [170]. Neurotrophic factor gene delivery aimed to enhance regeneration and sprouting via direct effects on neurons and their axons may also indirectly promote axonal growth and functional outcomes. Gliogenesis [171], Schwann cell proliferation [17], astrogliosis [41], endogenous neural stem cell differentiation, or neurogenesis [172] can be modified by viral delivery of growth factors or transcription factors. Thus, glial and inflammatory reactions represent additional avenues for genetic manipulations.

Conclusions

Gene therapy faces the same requirements for translation as any treatment in spinal cord injured individuals. These include efficacy in a feasible time window after injury, practicality, the need for robust preclinical, functionally relevant improvements in animal models, and ideally single rather than repeated interventions. In addition, many of the gene therapy approaches described in this chapter will likely require means to temporally control gene expression. Regulatable gene expression systems or vector/promoter systems that will spontaneously cease to express a gene of interest are needed for treatments that promote axonal growth, activate the intrinsic growth capacity, guide regenerating axons, or modify the cellular and extracellular matrix in the injured spinal cord. However, many molecular on–off switches that have been developed to date have not been sufficiently tested in long-term experiments or are immunogenic precluding their use in human subjects. The lack of such systems has slowed the progress in translating findings from bench to bedside. Adverse effects such as increased spasticity after long-term delivery of high

levels of BDNF [92, 93, 173] currently limit options for clinical trials of neurotrophic factors after SCI despite a good safety record in neurodegenerative diseases including Alzheimer's disease and Parkinson's disease. Thus, promising findings of gene therapy activating neuron-intrinsic regenerative programs and modifying the extrinsic environment in the acutely and chronically injured spinal cord have yet to be clinically translated. Ongoing advances in targeted genome modification and improved vector and promoter systems allowing for minimally invasive, localized, cell-specific, and regulated gene delivery will provide the chance to clinically address the complex and multifaceted nature of SCI.

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Chapter 9
Gene Therapy for Epilepsy

Thomas J. McCown

Abstract Focal epilepsies present an attractive target for viral vector gene therapy especially in those cases approved for surgical resection. A number of basic studies have identified potential therapeutic approaches ranging from neuroactive peptides and potassium channels to DREADD receptors. Although each approach exhibits clear therapeutic strengths, a number of concerns remain. Immune responses, variable cellular tropisms, off-target liabilities, and surgical infusion parameters must be considered in order to move these therapies to the clinic.

Keywords Adeno-associated virus • Epilepsy • Gene therapy • Neuroactive peptides • Potassium channels • DREADD receptors

Introduction

Epilepsy comprises one of the most prevalent neurological disorders throughout the world where the overall incidence is approximately 1 % [1]. A diagnosis of epilepsy arises when an individual experiences at least two unprovoked seizures more than 24 h apart, and fortunately in many cases current anti-seizure drug therapy proves effective. For example, current anti-seizure medication effectively controls seizures in approximately 70 % of people receiving optimal care. However, the remaining 30 % of patients do not achieve adequate seizure control with current anti-seizure drugs [2, 3]. Thus, in this year alone, nearly 150,000 new cases of epilepsy will be diagnosed in the United States, and of this number, approximately 45,000 will prove refractory to medication. Resective surgery provides an alternative treatment for this refractory population, but fewer than 10 % of patients with drug refractory epilepsy are considered for surgical resection [4–7]. More importantly, the proportion of drug-resistant epilepsies has remained the same even with the introduction of numerous new anti-seizure drugs over the last 20 years [8, 9]. Clearly a substantial

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need exists to develop novel, more effective treatments for this refractory population. Advances in viral vector gene therapy provide several promising alternatives.

When considering clinical translation, adeno-associated virus (AAV) vectors exhibit a range of properties that prove highly applicable to the treatment of focal epilepsies. In the CNS, AAV vectors support nontoxic, long-term transduction of neurons (see Ref. [10] for review). Not only can stable gene expression be achieved, but in most cases the vectors do not elicit an immune response. Recent discoveries of additional AAV serotypes have greatly expanded the capability to achieve differential vector spread within the CNS, as well as differences in the absolute gene expression per transduced cell [11, 12]. In general AAV2 does not spread very far from the injection area and supports moderate gene expression per transduced cell. In contrast, AAV5 spreads much further than AAV2, while AAV8 not only spreads further but supports a greater level of gene expression per cell [12]. However, upon direct injection into the CNS all three serotypes, as well as other serotypes such as AAV9 or AAVrh10, exhibit a dominant tropism for neurons in vivo. Thus, AAV viral vectors can achieve substantial long-term, stable gene expression in neuronal populations. These in vivo properties appear ideal for a specific epileptic population, drug refractory patients who have been approved for surgical resection. In these cases, extensive mapping studies will be conducted that identify the seizure focus and determine the extent of surgical resection. In most cases AAV vectors should prove sufficient to transduce the majority of the tissue scheduled for resection, but nonhuman primate studies still must be conducted to establish the appropriate infusion parameters and viral vector dosage. However, unlike other CNS gene therapies, the presence of an approved surgical resection procedure provides the perfect rescue option.

A number of AAV preclinical studies have investigated anti-seizure gene therapies using a variety of animal seizure models. From the expression and secretion of neuroactive peptides to the expression and optical activation of channelrhodopsins, a wide range of approaches have been pursued yielding positive preclinical results (see Refs. [13, 14] for reviews). In every case though, specific strengths that support clinical translation must be considered in the broader context of viral vector properties and potential adverse effects.

Modulatory Neuroactive Peptides

The earliest epilepsy-related gene therapy studies focused upon the neuroactive peptides, galanin and neuropeptide Y (NPY). A substantial literature previously established the anti-seizure efficacy of both neuropeptides which provided the basic rationale for this gene therapy approach [15–17]. Initially, Haberman et al. [18] combined the observation of galanin's robust anti-seizure activity in vivo with the fact that the fibronectin secretory signal sequence produced constitutive secretion of fibronectin from the cell. When the coding sequence for the fibronectin secretory signal sequence (FIB) preceded the coding sequence for the active portion of galanin (GAL), subsequent transduction with AAV2 vectors attenuated focal seizure

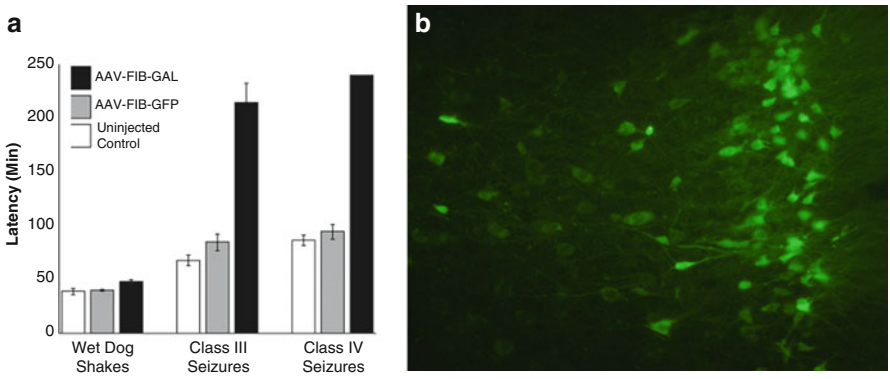

Fig. 9.1 The effects of AAV-FIB-GFP and AAV-FIB-GAL vectors on the expression of limbic seizure behaviors. Panel (**a**) shows that 7 days after infusion of AAV-FIB-GFP into the piriform cortex, kainic acid administration (10 mg/kg, i.p.) elicited wet dog shakes, class III and class IV seizure activity, with the same latency as in untreated controls. Seven days after infusion of AAV-FIB-GAL ($N=12$) into the piriform cortex, kainic acid administration elicited wet dog shake behaviors with the same latency as in untreated control animals. In contrast, the observed overall seizure activity was a brief class III seizure exhibited by only 1 of the 12 animals over the 240-min post-kainic acid observation period. None of the animals exhibited any class IV seizure behaviors over the entire 240-min post-kainic acid observation period. Panel (**b**) shows the expression of GFP after a similar AAV-FIB-GFP infusion into the piriform cortex (From [19]; figures used with permission from Nature Journals)

activity and prevented kainic acid seizure-induced cell damage in the hippocampus. These initial findings were extended by McCown [19] who showed that the FIB–GAL containing vectors essentially prevented kainic acid seizures (Fig. 9.1). Another study used AAV vectors to express galanin alone which reduced seizure severity but did not prevent seizure-induced cell death [20]. These findings established the anti-seizure efficacy of vector-derived galanin, but also illustrated the need to provide the appropriate trafficking information in order to achieve an optimal effect from the gene product.

Like galanin a number of studies have established that NPY attenuates seizure activity in vivo. For example, direct infusion of recombinant NPY significantly attenuates seizure activity [16, 17]. Therefore, Richichi et al. [21] expressed a preproNPY sequence that markedly reduced EEG seizures and delayed seizure onset. However, significant results required an AAV1/AAV2 chimeric virus in order to achieve higher levels of gene expression compared to AAV2. Subsequent studies further demonstrated anti-seizure effects by packaging the preproNPY cDNA into AAV1 capsids where expression was driven by a stronger promoter (chicken beta actin) flanked by a woodchuck post-regulatory element (WPRE) [22]. Clearly under these conditions the level of gene expression proved critical to a positive outcome. By using the same approach as Haberman et al. [18], Foti et al. [23] established that the expression and constitutive secretion of either NPY or the NPY Y2 receptor active fragment, NPY (13–36), significantly attenuated kainic acid-induced seizures.

Translation Considerations These studies established the effectiveness of vector-derived neuropeptides in attenuating seizure sensitivity in animal models of temporal lobe epilepsy. The evidence strongly supports therapeutic efficacy with regard to seizure attenuation, particularly on a localized basis. In light of these encouraging findings, however, several issues remain in order to facilitate a path toward clinical trials. First, the effectiveness depends upon the release of the vector-derived neuropeptide in the area of seizures and the local presence of the appropriate peptide receptor. For example if the transduced cells project to distal structures and a prepropeptide sequence is utilized, the actual release of the vector-derived peptide may occur outside the intended target. Secondly even if the peptides are released in the area of infusion, an effect depends upon the presence of the appropriate receptor. At least in the case of galanin and NPY, the appropriate receptors appear to be present in the hippocampus, the most common focus of intractable temporal lobe seizures. Also, it will be important to minimize the potential of off-target effects if the peptides are expressed in other brain areas, such as the hypothalamus. For example, expression and constitutive secretion of galanin or NPY in the hypothalamus could exert adverse effects on food intake, appetite, and emotional state. This concern can be ameliorated using receptor-specific peptide fragments such as NPY (13–36) (Foti et al. 2007) or galanin (2–11) [24] Finally, the emergence of numerous AAV serotypes must be considered in the context of off-target effects. AAV2 serotypes have been used in numerous CNS clinical trials without any evidence of toxicity, and even if AAV2 vectors leak into the ventricular system, distal structures will not be transduced. In marked contrast, serotypes such as AAV9 can transduce distal structures after CNS administration [25]. Because the hippocampus forms one wall of the lateral ventricles, the consequences of vector leakage into the ventricular system must be considered prior to clinical trials.

Another concern for in vivo expression of these neuroactive peptides involves the potential to impair normal behavioral or physiological processes. In the case of NPY, some research suggests that preproNPY expression does not lead to any apparent learning and memory, emotive, or locomotor deficits [26]. However, one study found that naive rats treated with AAV–NPY in the hippocampus showed transiently delayed hippocampal-based learning and attenuation of long-term potentiation (LTP) in CA1 [27]. Subsequent research by the same group showed that a kindling model of epilepsy similarly caused a compromise in both short-term synaptic plasticity and LTP, but additional treatment with the NPY vector did not further exacerbate the kindling-induced changes in hippocampal function [28].

Neurotrophic Factors

In addition to the neuroprotective and growth factor roles, studies have established that the neurotrophic factors, glial-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), and brain-derived neurotophic factor (BDNF) can influence seizure activity after viral vector gene transfer. Adenoviral vector overexpression of

GDNF prior to kainic acid administration reduces kainic acid-induced limbic seizure activity and partially protects against seizure-induced cell death [29]. Subsequently, Kanter-Schlifke et al. [30] reported that transduction of dorsal and ventral hippocampus with AAV–GDNF reduced the number of generalized seizures and shortened the seizure duration evoked by ventral hippocampal kindling. Furthermore, post-kindling treatment with AAV–GDNF led to an increased seizure threshold in these animals and reduced seizure frequency during rapid stimulation-induced status epilepticus.

Another growth factor approach involved the overexpression of both fibroblast growth factor-2 (FGF-2) and brain-derived neurotrophic factor (BDNF). Using a replication defective herpes simplex 1 vector, Paradiso et al. [31] transiently overexpressed both FGF-2 and BDNF in the hippocampus 4 days after pilocarpine treatment. The treated animals exhibited a significant reduction in hippocampus cell death, as well as a significant reduction in the severity, duration, and appearance of spontaneous seizure activity. However, if the vectors were infused after the appearance of spontaneous seizures, no effects were found. Thus, this approach appeared to influence epileptogenesis but not seizures per se.

Translational Considerations Viral vector-mediated GDNF expression directly influenced seizure sensitivity, but in all instances the level of suppression was partial. Conversely, co-expression of FGF-2 and BDNF influenced the course of epileptogenesis but had no effects on seizure sensitivity. Although in both of these instances the effects were significant, a number of factors diminish the likelihood for neurotrophic factor clinical translation. Currently, it cannot be envisioned that direct viral vector infusion into the CNS would precede the appearance of seizures, so trials to attenuate epileptogenesis are not practical. More importantly, a number of potential detrimental effects have been attributed to long-term expression of neurotrophic factors such as GDNF or BDNF [32]. These negative factors substantially reduce the translational potential for growth factor seizure gene therapy.

Adenosine

The endogenous compound, adenosine, exerts the ability to suppress seizure activity in vivo [33], so a number of studies have focused upon strategies to elevate local adenosine concentrations in the CNS. One component of this approach utilized AAV vectors to manipulate the astrocytic enzyme adenosine kinase (ADK) which catabolizes adenosine into 5′AMP upon adenosine cellular uptake [34, 35]. Spontaneous seizure activity occurs when AAV vectors overexpress ADK in astrocytes [36]. Conversely when AAV8 vector with an astrocyte-specific promoter overexpressed an ADK antisense in astrocytes, seizure activity was blocked in mice that were genetically modified to overexpress ADK [37].

Translation Considerations Although substantial evidence suggests that endogenous adenosine modulates seizure activity, the gene therapy approach was tested in

a model that was biased toward the outcome. Because the seizure activity emerged from an overexpression of ADK, a reduction of ADK activity would be expected to reduce the seizure activity. Unfortunately, studies have not attempted to test this gene therapy in other seizure models. Also, this approach requires astrocyte-localized gene expression which for AAV vectors has not proven very robust in comparison to neuronal transduction. Thus, at present the translational potential is quite low.

Ion Channels

The discovery of optogenetics has provided a novel advance in the control of ion flow within individual cells where light activation of channelrhodopsins alters ion flow within the cell. Thus, in neurons it proves possible to produce selective excitation or inhibition within a circumscribed neuronal network in vivo. Given this exquisite control of neuronal excitability, a number of studies have established the ability of optogenetics to control seizure activity. For example, Krook-Magnuson et al. [38] crossed two mouse strains to achieve excitatory channelrhodopsin expression in parvalbumin containing inhibitory neurons. When seizures were elicited with local kainic acid administration, light activation of the channelrhodopsin significantly attenuated the ongoing seizure activity. Similarly Ledri et al. [39] reported that in acute hippocampal slices selective activation of GABA inhibitory neurons suppressed epileptiform activity. Of more direct relevance to epilepsy gene therapy, Wykes et al. [40] used lentiviral vectors to transduce pyramidal neurons in vivo and express the chloride pump halorhodopsin in a neocortical focal model of epilepsy. Light activation of the halorhodopsin significantly attenuated the electrographic seizure activity within the seizure focus. From these findings the authors suggested that this technique could be used as an on-demand means to terminate seizure activity.

Another ion channel approach involved expressing the potassium channel Kv1.1 which reduces neuronal excitability [41]. A lentiviral vector was used to transduce pyramidal cells and express the Kv1.1 potassium channel in the same neocortical model of epilepsy employed for the optogenetic studies [40]. Not only did the expression of this potassium channel prevent the development of the epileptiform activity, but the Kv1.1 channel expression also significantly reduced previously established seizure activity.

Translation Considerations Although a powerful in vivo technique, optogenetics contains a number of significant concerns that preclude translation to the clinic. First, current methods of light activation can only influence highly circumscribed areas, so it seems unlikely that the small area of influence demonstrated in mice can be translated readily to the much larger areas of influence required to attenuate focal cortical seizures. A more important consideration, however, involves recent immunological findings. Samaranch et al. [42] recently reported findings in nonhuman primates that established a serious concern with regard to non-self protein expression in the CNS by AAV9 vectors. When AAV9 vectors were infused into the cisterna magna of the nonhuman primate and expressed GFP, the animals became

ataxic to the point that one had to be euthanized. Subsequent analysis revealed a significant immune response characterized by activation of astrocytes and microglia, upregulation of MHC-II, and toxicity to cerebellar Purkinje neurons. This immune response was attributed to the transduction of astrocytes which are antigen-presenting cells in the CNS. No such response was found for the same AAV9 administration which expressed human aromatic-amino acid decarboxylase. Thus, expression of non-self proteins in astrocytes can have serious adverse consequences. This liability is especially pertinent to epilepsy, because Weinberg et al. [43] demonstrated that the transduction pattern of AAV5 which is predominantly neurotrophic shifts toward more astrocyte transduction in a seizure milieu. Thus, AAV vector expression of the foreign opsin protein in a seizure focus could very likely cause a pathological immune response. Clearly, the optogenetic gene therapy approach contains significant risks that are incompatible with clinical translation.

In contrast, expression of the Kv1.1 potassium channel exhibits properties that have significant translational potential. Assuming that pyramidal cells can be transduced in the human neocortex, the properties of this ion channel would allow suppression of seizure activity without total disruption of overall neuronal activity. Certainly the preclinical evidence suggests that overexpression of this ion channel can attenuate focal seizure activity in the cortex. Furthermore, lentiviral vectors can transduce an area sufficient to influence seizures within a cortical focus that has been scheduled for resection. Before advancing, however, pyramidal cell specificity must be demonstrated in a nonhuman primate, and in the case of intractable temporal lobe epilepsy, success must be demonstrated in limbic seizure models prior to any attempt to translate this approach to those patients scheduled for temporal lobe resection.

DREADD Receptors

A recent novel approach to seizure suppression involved the use of a designer receptor exclusively activated by a designer drug (DREADD). These G-protein coupled receptors have been modified such that receptor activation requires the presence of a selective ligand, such as clozapine-*N*-oxide (CNO) (see [44] for review). Thus, viral vector-mediated transduction and expression of these receptors only influences cellular activity after the peripheral administration of CNO. Kätzel et al. [45] used an AAV5 vector to express the modified muscarinic receptor $hM4D_i$ in the motor cortex. Subsequent, local administration of either pilocarpine or picrotoxin caused both electrographic and motor seizure activity, but peripheral administration of CNO significantly attenuated this seizure activity. Similarly in a chronic model of focal cortical seizure activity expression of the $hM4D_i$ and subsequent CNO administration attenuated the seizure activity. Thus, this DREADD receptor approach exhibited the ability to attenuate seizure activity in vivo.

Translation Considerations This novel approach contains a number of properties that warrant consideration for viral vector seizure gene therapy. First the receptor exhibits enough similarity to the endogenous protein such that an immune response

to the vector-derived gene product seems unlikely. Also, receptor activation requires peripheral drug administration such that influence of the vector-derived gene product can be controlled peripherally. This peripheral control of the gene product provides an additional measure of safety particularly in light of long-term CNS gene expression. However, before clinical consideration can be considered, the overall magnitude of seizure suppression should be demonstrated in relevant chronic focal seizure models. For example, the actual decrease in picrotoxin behavioral seizures was 39 %. Thus, although significant, this magnitude of seizure suppression might be insufficient to warrant such an invasive clinical trial.

Conclusion

A number of gene therapy approaches have been investigated using viral vectors to express gene products that attenuate seizure activity in vivo. A major advantage to these approaches is the established safety of the AAV vectors in the CNS and the

Table 9.1 Summary

Approach	Original basis	Preclinical	Late preclinical[a]	Clinical
Adenosine	Acute, direct seizure suppression [33]	AAV8-mediated seizure reduction in mice with genetically reduced adenosine [37]	None	None
Neurotrophic factors FGF2, BDNF GDNF	Blocks kindling progression [46, 47] Delays amygdala kindling progression [48]	Lentiviral vector FGF2 and BDNF expression reduces seizure severity [31] AAV-GDNF reduces seizure number and duration [30]	None	None
DREADD receptors	Influence on neuronal excitability after drug (CNO) administration [44]	AAV-hM4Di expression and peripheral CNO administration attenuates cortical seizures [45]	None	None
Ion channels Kv1.1 potassium channel	Modulates neuronal excitability [41]	Lentiviral-Kv1.1 expression attenuates focal cortical seizures [40]	None	None
Neuroactive peptides galanin NPY	Both galanin and NPY attenuate seizure activity in vivo [16]	AAV-mediated galanin expression/constitutive secretion blocks seizure activity [18, 19] AAV-mediated NPY expression suppresses both acute and chronic seizures [21, 23, 26]	AAV-mediated NPY expression does not significantly alter learning and memory [28]	None

[a]Late preclinical studies focus upon overall toxicity or primate investigations

ability to rescue the transduced tissue by surgical resection. When considering potential immune responses, cell-specific requirements and overall efficacy, neuroactive peptides, and Kv1.1 potassium channels appear to have the best likelihood of future clinical translation (Table 9.1).

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Chapter 10
Translating Gene Therapy for Pain from Animal Studies to the Clinic

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Abstract The use of gene transfer techniques, designed to effect the continuous release of analgesic peptides, offers the possibility to treat what may otherwise be intractable pain. In this chapter, we review the biology underlying this approach, the results of preclinical experiments in animal models, the human trials that have been completed, and prospects for the near-term future.

Keywords Gene therapy • Inflammatory pain • Neuropathic pain • HSV • Enkephalin • GABA

Introduction

The first animal experimental studies suggesting that gene transfer might be used to reduce pain-related behaviors were published just over 15 years ago. In 1998, Michel Pohl and coworkers reported that the rat proenkephalin A (pEnkA) gene could be delivered to sensory neurons of the rat dorsal root ganglion using a herpes simplex virus (HSV)-based vector, and when expressed by a fusion promoter, consisting of the region upstream from the HSV LAT core promoter

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and elements of Moloney murine leukemia virus long terminal repeat (LTR), resulted in a substantial increase in enkephalin concentration in the dorsal horn of spinal cord [1]. This was followed soon thereafter by a report from Steve Wilson, David Yeomans, and Joe Glorioso using a related HSV-based vector to express human preproenkephalin (PENK) under the control of the human cytomegalovirus immediate early promoter that demonstrated an antihyperalgesic effect after sensitization of sensory afferents by topical application of capsaicin or dimethyl sulfoxide indicating altered responsiveness of both C and A∂ fibers to stimuli which would normally produce hyperalgesia [2]. The authors speculated that the vector employed "or a similar recombinant herpes virus may be useful for treatment of chronic pain in humans" because, they noted, "hyperalgesia, which may be important in establishing and maintaining neuropathic and other chronic pain states, was selectively blocked by infection with this proenkephalin-encoding virus." They went on to point out that "advantages of this type of gene therapy would include precise anatomical targeting of the specific nociceptors transmitting pain impulses, the lack of systemic opioid adverse effects, and a long (weeks to months) duration of action."

About the same time, Mike Iadarola and colleagues reported that an adenovirus-based vector encoding a secretable form of the endogenous opioid β-endorphin injected intrathecally effectively transduced meningeal cells of the pia mater and that β-endorphin was released into the CSF from transduced cells, while having no effect on withdrawal from a thermal stimulus in normal animals, this treatment substantially attenuated the exaggerated withdrawal response after injection of carrageenan into the paw [3]. The authors suggested that "the simplicity of this meningeal–paracrine gene therapy approach, rapidity of expression, ease of application, and apparent lack of side effects open the possibility of a more general clinical utilization…the basis for a novel therapy for pain control."

These three studies set the stage for the principal approaches that have been considered for translating gene transfer to a useful therapy for chronic pain in patients. Readers of this book should require no convincing that chronic pain is an important clinical problem that produces a substantial adverse impact on quality of life for a large number of people, resulting not only in major medical costs but producing a general adverse economic impact on society. The focus of this review will be on the challenge inherent in moving a novel therapy for pain from preclinical animal studies, through human clinical trials, and ultimately into widespread use in patients. The extensive literature reporting the effects of different animal models and gene transfer vectors in experimental studies of pain has been reviewed recently [4], and the reader is referred to that review for a comprehensive overview. After the first initial studies 15 years ago, subsequent work has largely focused on examining the effect of different gene products in different animal models of chronic pain that are considered (more or less) to mimic human conditions.

Animal Studies of Gene Transfer for Pain

DRG Transduction by Skin Inoculation

One key feature of the HSV-based approach is that DRG neurons can be transduced noninvasively by skin inoculation (Fig. 10.1). As the first-order neuron in the path of nociceptive neurotransmission from the periphery to the brain, DRG represent an important target either for strategies that would directly silence these neurons or for expression of substances that can be released to modulate nociceptive neurotransmission at the synapse in the dorsal horn between primary afferent and projection neurons. HSV-based vectors expressing preproenkephalin have been demonstrated to reduce pain-related behaviors in the delayed phase of the formalin test [5], a polyarthritis model of inflammatory pain [6], the osteolytic sarcoma model of cancer pain [7], the spinal nerve ligation model of neuropathic pain [8], pertussis toxin-induced thermal hyperalgesia [9], the infraorbital nerve ligation model of trigeminal pain [10], pain related to inflammation of the pancreas [11, 12], a rodent model of monoarthritis [13], a diabetic model of neuropathic pain [14], and a model of interstitial cystitis/painful bladder syndrome [15]. The effect of the enkephalin-expressing vector in reducing hyperalgesia has been confirmed in a primate model [16].

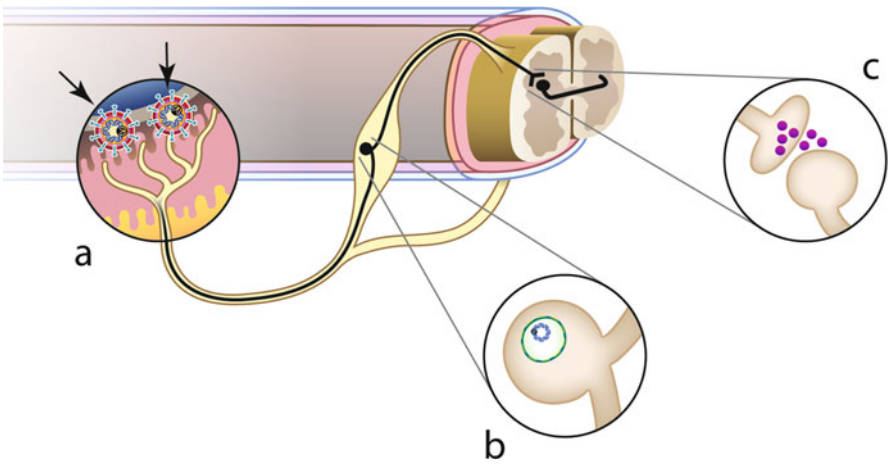

Fig. 10.1 (**a**) Non-replicating HSV vectors are injected into the skin (*arrows*, **a**) and taken up by nerve terminals. (**b**) The vector genome is retrogradely transported to the cell body in the DRG where the DNA establishes a persistent state as an intranuclear, episomal element. (**c**) Peptides coded by the vector genome are synthesized and transported to the nerve terminal where they may be released from the nerve terminal to act on second-order neurons in the pain pathway

An HSV-based vector expressing glutamic acid decarboxylase (GAD) to effect the release of the inhibitory neurotransmitter γ aminobutyric acid (GABA) reduces pain-related behaviors in rodent models of neuropathic pain resulting from spinal nerve ligation [17] or from painful diabetic neuropathy [18] and reduces pain-related behaviors [19] and detrusor overactivity [20] in a model of spinal cord injury. Unlike the preproenkephalin gene product that contains a leader sequence to direct transport into vesicles for appropriate processing and release, transgene-mediated GAD remains cytoplasmic and the resulting GABA is released from the transduced nerve terminals not by vesicular release, but rather through the GABA transporter (GAT1) that responds to the high concentration of intracytoplasmic GABA by functioning in reverse, to release GABA into the extracellular space [21].

Other genes that have been transferred by HSV vectors to produce excreted peptides to modify pain-related behaviors include glial derived neurotrophic factor (GDNF) in the SNL model of neuropathic pain [22]; interleukin 4 (IL-4) in the SNL model of neuropathic pain [23] and a bladder pain model [24]; a soluble fragment of the p55 tumor necrosis factor α receptor in the spinal nerve ligation model of neuropathic pain [25], an HIV nucleoside reverse transcriptase inhibitor neuropathy model [26] and HIV gp120 neuropathic pain model [27], and a bladder pain model [28]; IL-10 in the formalin model of inflammatory pain [29] and spinal cord injury pain [30]; and endomorphin 2 in neuropathic pain and in the CFA-induced model of inflammatory pain [31, 32].

One would anticipate that HSV-mediated gene transfer would be most effective when the transgene product is released because of the opportunity for paracrine effects to produce desired therapeutic results in nearby cells that had not been transduced by the vector. Nonetheless, HSV-mediated gene transfer of interfering RNAs to knock down expression of a pronociceptive gene product in peripheral neurons has also proven to be effective, as demonstrated by the effects of knockdown of the voltage-gated sodium channel NaV1.7 in inflammatory hyperalgesia [33] and in painful diabetic neuropathy [34]. More complicated approaches such as expression of a mutated glycine receptor have also been reported [35].

Gene Transfer by Intrathecal or Intraneural Delivery

Intrathecal injection of adenovirus has been used to deliver IL-2 in a model of neuropathic pain [36], the glial glutamate transporter (GLUT1) in inflammatory and neuropathic pain [37], the CBD peptide as a calcium channel inhibitor in neuropathic pain [38], a NaV1.3 shRNA in neuropathic pain [39], and endomorphin-2 in neuropathic pain [40]. Others have used adeno-associated virus (AAV) to deliver BDNF [41], IL-10 [42], or β-endorphin or IL-10 [43] in models of neuropathic and inflammatory pain, and lentivirus to deliver an interfering RNA [44]. Similar results have been reported using plasmid or plasmid combined with polymer-based delivery of anti-inflammatory cytokines in models of neuropathic pain [45–48]. Importantly, in at least one of the reports [43] intrathecal injection of an AAV serotype 8-derived vector was found to result in substantial infection of DRG neurons.

An alternative to skin inoculation with herpes vectors or intrathecal inoculation with other gene transfer vectors is direct injection into the DRG or nerve to transduce DRG neurons [49, 50] or into the trigeminal ganglion (TG) to transduce TG neurons [51]. In rodents, different serotypes of AAV produce infection of different classes of neurons (large diameter, producing myelinated fibers, vs. small diameter, producing unmyelinated fibers for instance). A related, less invasive alternative is direct intraneural injection to infect DRG neurons presumably by retrograde axonal transport, which can be used to express inhibitory neurotransmitters [52], inhibitory neurotransmitter receptors [53], or light-sensitive ion channels that can be activated by illumination through the skin [54].

Gene Delivery by Intraparenchymal Injection

Direct intraparenchymal injection of gene transfer vectors is an effective means to express gene products within the central nervous system, and injections into nuclei in the brain [55], brainstem, and spinal cord [56] have all been used in animal models to explore the role of specific nuclei for gene products in the phenomenon of pain perception. Of the three routes of gene delivery (skin inoculation, intrathecal or intraneural inoculation, and intraparenchymal injection), the latter in our view is the least likely to be translated to clinical application in the foreseeable future. Alternately, vectors can be injected directly into the end organ at the site causing pain, as has been demonstrated in a model of pancreatitis using an HSV-based vector [11, 12], and in models of arthritis using HSV [13] and lentivirus-based vectors [57].

Translation to Clinical Trial

General Considerations

The first question that needs to be addressed as one considers moving from preclinical animal models to the clinic investigations is to determine the appropriate pain patient population for an innovative gene therapy trial. While severe pain is disabling and often resistant to treatment, pain itself is not a fatal condition. Thus, even though a patient is suffering from pain that does not respond to standard of care therapy, the patient may not be appropriate for a treatment that involves permanent gene transfer, even if that treatment were proven to be effective in relieving pain. At a practical level, it is necessary first to identify an appropriate population for initial clinical testing of the gene therapy platform, typically in a Phase 1 trial. However, moving forward, a viable therapy will also require a large enough population of potential patients to make the treatment commercially viable. Thus, it is necessary to consider not only an initial safety study and subsequent proof-of-concept studies in

patients, but to also have a clear corporate development plan and target product profile (TPP) that will ultimately guide the therapy through clinical trials and into the marketplace.

The second question that needs to be considered is: what type of pain is the gene therapy designed to treat? What has not been discussed above is that in both humans and in animal models, there are significant and important molecular, biochemical, and electrophysiologic differences between inflammatory (nociceptive) pain, neuropathic pain, cancer pain, and other pain syndromes. Whether these known, and likely many unknown, differences are reflected in each individual patient is also an important factor to consider. The formalin test for instance is very accurate in predicting the morphine equivalent of novel opiate compounds in the treatment of inflammatory pain, but has not been as useful in predicting the response of patients to compounds acting through other mechanisms for the treatment of pain. There is also a deficiency in evidence to demonstrate that drugs that reduce pain-related behaviors in models of neuropathic pain in rodents are specifically effective in neuropathic pain in humans. But given the current state of the art, it would seem both reasonable and prudent to have preclinical evidence appropriate for the type of pain being treated prior to moving into clinical trials in patients.

Other issues to consider, such as whether the approach is appropriate for pain that is localized to one or more parts of the body, or might be applied to pain that affects patients more diffusely will depend on a combination of the rationale underlying the gene therapy approach, the preclinical data, and the clinical indication. And finally, there are practical issues that will determine whether gene therapy for pain is a viable treatment. For what indication(s) will the FDA approve the treatment? How many patients fit into that category? How heterozygous is the patient population in the chosen indication? Can the vector be produced at a cost that will make the treatment competitive with other available options? Using the developed TPP, what indication should first be tested clinically for the therapy? Ultimately, can the treatment be provided in a manner that would allow widespread use in the community, or would it be restricted to specialized medical centers? In the following sections, we describe the path we have taken to clinical trials of HSV-based vectors for the treatment of pain.

Preproenkephalin for Inflammatory Pain

The first HSV vector that we utilized in a clinical trial is a replication-defective HSV-based vector expressing human preproenkephalin (PENK). In preclinical animal studies, we initially demonstrated that an HSV vector rendered replication defective by the deletion of the gene coding for immediate early gene product ICP4 and expressing human preproenkephalin gene under control of the human cytomegalovirus immediate early promoter (HCMV IEp) reduced pain-related behaviors in the delayed phase of the formalin test of inflammatory pain [5], in the spinal nerve ligation model of neuropathic pain [8], and in the osteolytic sarcoma model of cancer pain [7].

In contrast to testing of novel small molecules where the Phase 1 dose-finding and safety trials are conducted in normal volunteers, Phase 1 gene therapy trials are typically carried out in patients. On theoretical grounds we were confident that delivery of the platform vector into the skin would be safe, because the number of vector particles injected into the skin would be orders of magnitude lower than the number of wild-type viral particles present in a typical cold sore. The safety of HSV vectors is also supported by extensive prior studies of replication competent/attenuated oncolytic HSV-based vectors that had been injected directly into the tumor of cancer patients and have advanced to Phase 3 clinical trials, or delivered peripherally as potential vaccines aimed at generating protective anti-HSV immunity (all of which have thus far failed in clinical trials). Even with this observed safety of replicating HSV vectors, our platform would be the first time a replication-defective HSV vector would be injected into the skin specifically to express potentially therapeutic genes directly in the targeted DRG. Therefore, we chose to carry out the Phase 1 safety dose-finding study in patients with intractable pain from terminal cancer.

The first step in moving forward toward a clinical trial was a public presentation to the Recombinant DNA Advisory Committee of the NIH which took place in June 2002. For this first clinical trial we proposed to employ a vector that utilized the HCMV IEp to drive transgene expression. The HCMV IEp is a transient promoter that drives expression of a transgene for a period of weeks/months in animals before the endogenous HSV latency mechanisms repress expression in vivo. We reasoned that this duration of expression should be sufficient to detect a clinical effect, and for this first Phase 1 trial represented an additional safety feature in that if adverse effects related to enkephalin expression were to occur those effects could be blocked by administration of naloxone until transgene expression would be naturally silenced.

Characterization of the Vector

1. Construction of the preproenkephalin-expressing HSV vector (NP2, now referred to as PGN-202) and complementing cell line. The NET (NEuronal Therapeutics) vector platform was constructed from an HSV genome engineered to be deleted for both the essential immediate early (IE) HSV genes ICP4 and ICP27 as well as for the UL55 coding region (Fig. 10.2). In addition truncations of the promoters for IE genes ICP22 and ICP47 removed their IE promoter nature, thereby constraining expression of these genes to complementing cells. The PENK transgene was inserted into the deleted essential ICP4 locus. Using this platform, two independent illegitimate recombination events would be required to generate a replication competent vector during manufacturing (which we have never detected). Further, the placement of the transgene into an essential gene locus mitigates the possibility of generating a replicating vector capable of expressing the transgene. The manufacturing cell line was generated by stably adding the HSV genes ICP4 and ICP27 individually into the genome of ATCC Vero (African

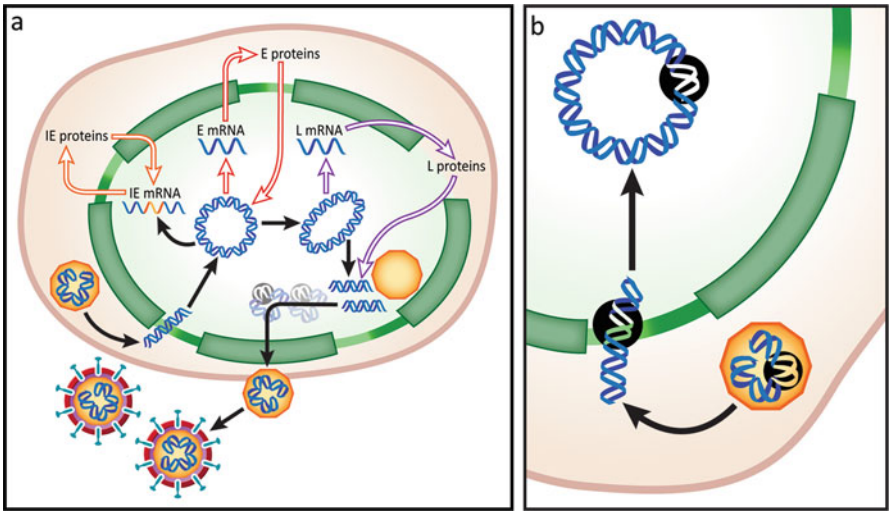

Fig. 10.2 (**a**) Wild-type HSV replication is characterized by a rigidly ordered temporal cascade that begins with the expression of immediate early (IE) genes, some of which are essential for the subsequent expression of early (E) and late (L) genes leading to the production of new virus particles. (**b**) Deletion of just one essential IE gene (indicated by *black circle*) renders the recombinant incapable of replication in any but the specific complementing cell line engineered to express the missing gene (or genes) from the cellular genome. The vector used for human trials fails to express 4 of the 5 IE genes

green monkey kidney) cells, and the resulting cell line was tested for the ability to complement replication-defective HSV production. The replication-defective HSV backbone was engineered in coordination with the complementing cell line in order to eliminate all overlaps between sequences flanking deleted essential IE genes in the vector and the IE gene sequences in the cell line in order to avoid recombination events that could generate replicating virus. The HSV vector backbone and complementing cell line was discussed with and approved by the FDA for cGMP production. NP2 was constructed by recombining a targeting plasmid containing the ICP4 flanking sequences and the human PENK expression cassette with a parental HSV vector backbone containing a GFP transgene in the ICP4 locus. The resulting recombinant was purified through three rounds of single plaque isolation and amplified into a seed stock. The seed stock was put through and passed a series of assays designed to confirm PENK expression, establish titer, demonstrate sterility, and establish endotoxin levels. The seed stock was then expanded and purified to produce material for GLP toxicology and biodistribution studies.

2. Toxicology of NP2. Following a pre-IND discussion with the FDA's Center for Biologics, Evaluation and Research (CBER), Office of Cellular, Tissue and Gene Therapies (OCTGT), a toxicology study using NP2 was performed

in compliance with US FDA (21 CFR Part 58). Four groups of 80 mice (40 male, 40 female) were dosed on study day 0 with either PBS or NP2 at 1×10^3, 1×10^5, or 1×10^7 PFU/animal, and 10 mice/sex/group/time point sacrificed on days 1, 7, 28, and 91. There was no evidence of treatment-related adverse effects as judged by clinical observations, body weight, or food consumption. Clinical pathology revealed no significant changes in hematology or clinical chemistry parameters at each time point. There were no treatment-related abnormalities in organ weights and macroscopic pathology. Histopathology examination revealed no test article treatment-related microscopic changes. Based on the predefined parameters of the toxicology study, administration of the test article according to the conditions of this study was well tolerated with no significant toxicity with the highest dose confirmed as the No Observed Adverse Effect Level (NOAEL).

3. Biodistribution of NP2. A total of 2400 tissues were harvested from the in-life phase of the biodistribution study. Using vector-specific primers, a GLP-validated QPCR assay was developed and DNA extraction efficiencies of target tissues performed. All tissues from the day 1 and day 7 cohorts were examined, as well as tissues from 10 animals of each sex from the control (PBS) group. In agreement with FDA guidance, because vector was observed only at the injection site, underlying muscle, and associated DRG on day 1 and day 7 samples, only these tissues were analyzed in the day 28 and day 90 cohorts. A total of 1180 tissue samples were evaluated. Of these, only samples from the injection site, underlying muscle, and associated DRG were found to have quantifiable vector sequences. There was no quantifiable dissemination to any other tissue. The FDA accepted the GLP biodistribution study as indicating that the vector platform is acceptably limited in distribution to the site of injection and the innervating DRG.
4. Production and certification of the master cell bank (MCB) and master viral bank (MVB). Prior to MCB production, a seed stock of complementing cells passed tests for sterility, mycoplasma, and endotoxin. A cGMP MCB of >200 vials with 1.0×10^7 cells/vial was produced at our GMP contract manufacturing organization (CMO) and tested for viable cell recovery, sterility, and mycoplasma and other standard safety and identity tests at external contract research organizations (CROs). For the NP2 vector, the seed vector stock was amplified at our CMO into an MVB using MCB cells. Final identity of the NP2 MVB was confirmed, according to guidance received from the FDA by a panel of tests including whole genome sequencing and a panel of safety, identity, and strength/potency testing parameters that constitute the Certificate of Analysis (COA).
5. GMP production of NP2 vector for human trial. NP2 was produced at our CMO using a proprietary multistep manufacturing and purification process. Sufficient GMP NP2 was produced and passed a final panel of safety, identity, and potency assays as required by the FDA to proceed with human clinical trials.

Clinical Trials of the Enkephalin-Expressing Vector

We conducted a multicenter, open-label dose-escalation, Phase 1 clinical trial of NP2 (now PGN-202) in subjects with intractable focal pain caused by cancer. NP2 was injected intradermally into the dermatome(s) corresponding to the radicular distribution of pain. The primary outcome was safety. As secondary measures, efficacy of pain relief was assessed using an 11-point Likert (0–10, 0 no pain and 10 worst pain) numeric rating scale (NRS), the Short Form McGill Pain Questionnaire (SF-MPQ), and concurrent opiate usage. Ten subjects with moderate to severe intractable pain scoring at least five on the NRS pain scale despite treatment with more than 200 mg/day of morphine (or equivalent) were enrolled into the study. Treatment was well tolerated with no study agent-related serious adverse events (SAEs) observed at any point in the study. Subjects receiving the low dose (1×10^7 PFU) of NP2 reported no substantive change in pain. Subjects in the middle (1×10^8 PFU) and high (1×10^9 PFU) dose cohorts reported pain relief as assessed by NRS and SF-MPQ. In summary, treatment of intractable pain with NP2 was well tolerated. There were no placebo controls in this relatively small study, but the dose-responsive analgesic effects suggested that NP2 may be effective in reducing pain and warranted further clinical investigation [58].

Following the completion of the Phase 1 study, we carried out a randomized, double-blind, placebo-controlled, multicenter Phase 2 clinical trial to investigate the impact of NP2 in patients with intractable pain due to malignancy. The primary endpoint of this study was change in the average NRS pain score (0–10) from the pretreatment period to the posttreatment period (day 3 to day 14 post-study drug administration) between NP2 and placebo-treated subjects. In comparison to the Phase 1 study, this Phase 2 study increased the qualifying pain score from an NRS pain score (5–7), increased the number of dermatomes to be treated (2–4), and allowed concomitant chemotherapeutic treatment with a dose of 3×10^8 PFU. In addition, subjects were allowed to receive two additional open-label doses of NP2, separated by 4–10 weeks, following the blinded portion of the study. A total of 33 patients were randomized with 30 patients (15 active, 15 placebo) included in the modified intent to treat (mITT) population. The primary therapeutic endpoint of the study was not met in this small exploratory Phase 2 study. Importantly, as in the Phase 1 study, the treatment in the Phase 2 trial was well tolerated. Details of the study design, results, and follow-up data will be provided in a future publication.

Clinical Trial of HSV-GAD in Neuropathic Pain

Because there is substantial evidence that implicates reduced spinal GABAergic inhibition in neuropathic pain [59, 60], we constructed a vector expressing GAD to produce GABA in transduced cells. In the T9 hemisection model of central

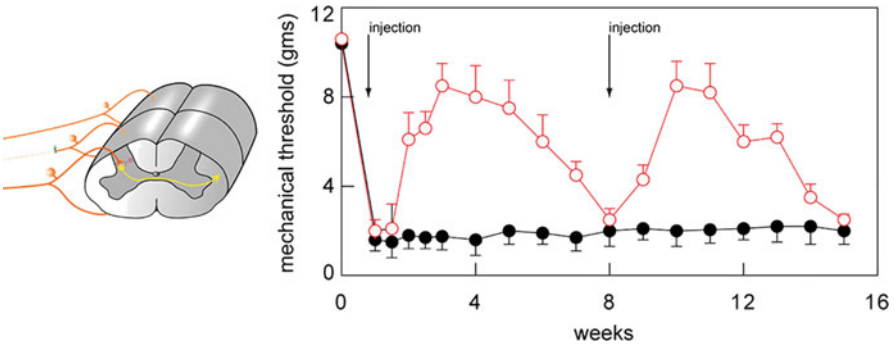

Fig. 10.3 In the selective L5 spinal nerve ligation model of neuropathic pain (*left*), subcutaneous injection of the GAD-expressing vector 1 week after nerve ligation substantially reverses mechanical allodynia. The effect persists for about 6 weeks, consistent with the time course of expression driven by the HCMV IEp from the context of the HSV vector genome, and is reestablished by reinoculation of the vector at 8 weeks

neuropathic pain resulting from spinal cord injury, subcutaneous inoculation of a GAD-expressing vector in the feet resulted in a substantial reduction in mechanical allodynia and thermal hyperalgesia in the hind limbs [19], an effect that was blocked by intrathecal injection of bicuculline or phaclofen [19]. In the SNL model of neuropathic pain, subcutaneous inoculation of the GAD-expressing vector 1 week after SNL produced a substantial antiallodynic effect that peaked about 2 weeks after inoculation and persisted for 6 weeks [17] (Fig. 10.3). The antinociceptive effect of vector-mediated GABA expression in these neuropathic pain models was substantially greater than that of vector-mediated enkephalin or endomorphin release. Like the antiallodynic effect of transgene-mediated enkephalin, the antiallodynic effect of the GAD-expressing vector waned over a time course of weeks, but was reestablished by reinoculation.

Based on the preclinical data, we went to the RAC at NIH in March 2009 with a proposal for a clinical trial of HSV GAD, and subsequently completed a pre-IND meeting with the FDA and achieved concurrence on IND enabling preclinical animal studies, on manufacturing and testing parameters, and on the clinical trial design. Similar to the PENK-expressing vector, the NET GAD-expressing vector (PGN-305) is a replication-defective HSV-1 recombinant modified as follows: (1) complete deletions of the viral ICP4, ICP27 (UL54), and UL55 genes; and (2) insertion of a human cytomegalovirus (HCMV) immediate early promoter-driven human glutamic acid decarboxylase (GAD65) expression cassette within both copies of the deleted ICP4 loci. The extent of the ICP4 deletion results in the removal of the upstream promoter sequences of the immediate early viral genes: ICP22 and ICP47. Utilizing the clinical manufacturing schema, we produced a PGN-305 seed stock, a toxicology lot, and a GMP MVB. A GLP toxicology/biodistribution study was performed at a

preclinical animal CRO. Rats were dosed by single (1×10^5 or 2×10^7 PFU per paw) or multiple (2×10^7 PFU per paw, 3 times over 2 weeks) subcutaneous injections in the plantar surface of the paw. There were no PGN-305-related changes in clinical signs, body weights, body weight changes, food consumption, clinical pathology parameters (hematology, coagulation, and clinical chemistry), and gross necropsy findings. Rats treated with repeat doses of PGN-305 did not have elevated anti-GAD65 Ab concentrations compared to vehicle controls or rats treated with one dose of PGN-305. Based on these results, the NOAEL was determined to be 2×10^7 PFU/paw, the highest dose tested.

Our proposed clinical trial includes two combined phases designed to evaluate the safety and efficacy of intradermal delivery of PGN-305 in subjects with painful diabetic neuropathy affecting the legs. The Phase 1 component is an open-label dose-escalation trial to evaluate safety of 3 escalating PGN-305 doses and determine the maximum tolerated dose (MTD). The Phase 2a component of the trial is a multicenter, randomized, double-blind, placebo-controlled trial that compares the MTD to placebo for further evaluation of safety and efficacy. Potential participants will be identified from patients seen in clinics at the participating sites having (1) Type 2 diabetes complicated by neuropathy confirmed by a score of ≥ 3 on the Michigan Neuropathy Screening Instrument (MNSI); (2) painful diabetic neuropathy with pain primarily in the legs for at least 6 months, with average daily pain score over 21 days screening period (with at least 14 days NRS assessments completed) ≥ 5 on the 0–10 NRS despite treatment with standard pain medications; and importantly, preserved nerve fibers in the skin of the lower leg ($\geq 2.5/mm^2$) determined by punch biopsy. The final inclusion and exclusion criteria will not be confirmed until just prior to filing of the IND.

For the dose-escalation safety portion of the study, PGN-305 will be delivered at doses of 0.5×10^8, 0.5×10^9, or 0.5×10^{10} PFU per leg and both legs will be dosed per subject; thus the total dose per participant will be 1×10^8, 1×10^9, or 1×10^{10} PFU cohorts. The drug will be injected intradermally in approximately 20 sites (100 μl per site) distributed over each lower leg from just above the ankle to just below the knee. For the efficacy portion of the trial, the dosing (route, volume) will be the same except that only the maximum tolerated dose of PGN-305 will be used and subjects will receive injections of PGN-305 or placebo delivered in an identical volume and number of injections.

The primary safety outcome for both the Phase 1 and Phase 2a stages will be assessed by careful evaluation of adverse events and serious adverse events. The primary efficacy variable for the phase 2a stage of the study will be changes in the average daily NRS pain score from baseline to the average daily NRS score of days 3–14 post-dosing. Secondary efficacy variables will include change in average daily NRS from days 14 to 28 post-dosing, change in the short form McGill pain questionnaire (SF-MPQ) score, change in Chronic Pain Sleep Inventory, change in the SF-12 physical component score and mental component score, and the proportion of subjects meet a 30 % reduction in the average daily numerical rating of pain. We anticipate initiating the study and enrolling patients in early 2015.

Future Directions

The first clinical trials completed with PGN-202 expressing PENK and the first clinical trial proposed for PGN-305 expressing GAD utilize the HCMV IEp to drive transgene expression because of the natural silencing of gene expression as a safety feature for early-stage trials. However, an important characteristic of our HSV vector technology includes long-term transgene expression from the episomal vector genome. Utilizing the HSV latency-associated (LAP2) promoter we have been able to demonstrate prolonged biologically active transgene expression up to 6 months after inoculation [61, 62], representing the duration of the experiments. Because LAP2 produces what appears to be lifelong expression of the latency-associated transcripts in natural infection, we doubt that 6 months is the limit of LAP2-driven transgene expression. More recently, we have shown that using the LAP2 promoter to drive a tet-on transactivator and with the transgene under the control of a minimal promoter linked to a tet-responsive element allows for expression in the DRG to be regulated under the control of oral administration of doxycycline [63]. Thus, if either or both vectors were to prove effective in the treatment of pain Phase 1/2a trials, there are options for achieving long-term or regulatable expression as we advance the HSV-based NET platform forward through clinical development toward the market.

In another line of work exploring HSV-mediated gene transfer to the DRG, we have demonstrated in a number of different preclinical models of polyneuropathy that HSV vectors expressing neurotrophic factors and delivered to the DRG by skin inoculation are effective in preventing nerve degeneration caused by drug intoxication, chemotherapeutic agents, or diabetes [62, 64–66]. In a separate project, we are proceeding forward toward a clinical trial to determine whether treatment with a vector expressing neurotrophin-3 can prevent the development of chemotherapy-induced neuropathy in patients receiving high dose chemotherapy for the treatment of cancer.

Concluding Thoughts

It is not uncommon in the current climate, particularly since NIH grant review has placed ever greater emphasis on “significance” and the implications of research proposals to have implications for treating human disease, to see basic science discoveries described in terms of their potential to be translated into novel treatments for disease. But what we have found in our experience is that the path from preclinical animal studies to the development of a human treatment is neither simple nor straightforward. Our group has been working with HSV-based vectors since 1989 and presented the proposal for our first clinical trial to the RAC in 2002. We have completed two clinical trials, dosed more than 40 subjects with some of the subjects receiving up to three doses, and to date no study agent serious adverse

events have been observed. Trials of novel treatments designed to reduce pain have the advantage that the primary readout (the patient's perception of their pain) is immediate and continuous, thus allowing for relatively short studies. The disadvantage of studying treatment for pain is the substantial placebo effect that is not limited to gene therapy trials but has frustrated the larger clinical pain research community [67, 68]. We anticipate that within the next 3–5 years we should be able to determine whether the approach of HSV-mediated gene transfer using the NET platform to treat indications such as pain or the prevention of neuropathy will introduce a viable alternative for the treatment of these conditions.

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Part II
Cellular Approaches

Chapter 11
Stem Cells for Parkinson's Disease

Andrés M. Bratt-Leal and Jeanne F. Loring

Abstract Parkinson's disease is a neurodegenerative disease primarily affecting the dopaminergic neurons in the midbrain. In this chapter, we discuss several aspects of Parkinson's disease that make it a worthwhile candidate for a stem cell-based therapy. Evidence that increasing the supply of a single neurotransmitter, dopamine, can significantly alleviate symptoms, a previous history of cell therapy, and robust protocols for generating authentic stem cell-derived dopaminergic neurons all offer a compelling case for cell therapy. We discuss the evolution of dopaminergic differentiation from stem cells, including supporting preclinical studies of efficacy in rodent and nonhuman primate models. We also highlight several clinical trials using allogeneic or autologous cells that are in progress or preparation by groups around the world.

Keywords Parkinson's disease • Stem cells • Cell therapy • Dopaminergic neurons • Induced pluripotent stem cells • Differentiation

Introduction

Parkinson's disease (PD), first described by James Parkinson in the early nineteenth century, is a neurodegenerative disorder that primarily targets dopaminergic (DA) neurons in the *substantia nigra pars compacta*. PD is the second most prevalent neurodegenerative disorder behind Alzheimer disease and affects over 1 % of the population over the age of 60 [1] and an estimated 7–10 million people worldwide. DA neurons in the *substantia nigra* innervate the striatum, where the neurotransmitter

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dopamine is released. Loss of proper dopamine regulation can lead to multiple symptoms, including motor symptoms (bradykinesia, tremor, and speech problems) and cognitive decline. It is estimated that over 50 % of the DA neurons in the *substantia nigra* have already been lost by the time a correct diagnosis is given [2]. While other neurons are involved, the A9 midbrain neurons appear to be the most susceptible to the death, especially early in the disease progression. There are many factors that have been identified as contributors to the development of PD, including environmental toxins and genetic susceptibility. Even so, the vast majority of PD diagnoses are still classified as idiopathic with unknown pathogenesis.

Advances in genomics have increased our understanding of genetic components of the disease, and currently there are 28 genes that are associated with an increased risk of PD. Mutations in these genes affect four general pathways involved in DA neuronal loss: synaptic neurotransmission, endosomal trafficking, lysosomal autophagy, and mitochondrial metabolism [3]. The first mutation to be associated with PD was discovered by studying a family with high incidence of the disease [4]. A mutation was found in the *SNCA* gene, which encodes for α-synuclein, a molecule that had already been identified as a major component of Lewy bodies, the protein aggregates associated with PD pathohistology. Similarly, mutation of the leucine-rich repeat kinase 2 (*LRRK2*) results in an increased risk of developing PD, but its penetrance is ethnicity specific. Though the exact mechanism is not known, common *LRRK2* mutations associated with an increased risk of PD, including the G2019S substitution, generally increase kinase activity of the LRRK2 enzyme. In vitro models of neurons carrying the G2019S mutation suggest that the mutation results in increased damage in the mitochondrial genome [5]. While highly penetrant variants were discovered by studying patients with familial forms of the disease, genome-wide-association studies (GWAS) have identified many more variants by genomic analysis of thousands of affected individuals. Most of the variants are not considered to be causal, but rather increase risk for the disease. The current dogma is that in most cases, environmental insult combines with genetic susceptibility to cause the loss of DA neurons. The genetic associations with PD raise an interesting issue about autologous vs. allogeneic therapies; because of the higher risk of DA neuron degeneration, autologous transplants may not be appropriate for patients carrying high-risk gene variants.

In the 1960s it was discovered that dopamine depletion was a primary cause of PD-associated motor symptoms, including bradykinesias. This led to trial of dopamine replacement therapy, the remarkable results of which led to FDA approval in 1970, and it is the standard of care even today. Therapy is usually given in the form of oral levodopa (L-DOPA). L-DOPA is the direct precursor to dopamine and is capable of crossing the blood–brain barrier. L-DOPA is delivered concurrently with a dopa-decarboxylase inhibitor which blocks conversion of L-DOPA to dopamine but does not cross the blood–brain barrier, thereby blocking dopamine synthesis outside the central nervous system. The L-DOPA plasma half-life when delivered alone is less than one hour, but is extended with simultaneous delivery of a dopa-decarboxylase inhibitor and/or a catechol-*O*-methyltransferase inhibitor, both of which block peripheral metabolism of L-DOPA. Early in the disease progression,

dopamine replacement is very well tolerated and effective in controlling motor symptoms. As the disease progresses, larger doses are required and the resulting improvement in symptoms is lessened, delayed, and can become unpredictable. It is hypothesized that prolonged treatment with oral L-DOPA can cause further damage to DA neurons through the pulsatile nature of bolus dose stimulation [6], which has led to the development of continuous release mechanisms such as transdermal patches [7] or continuous intraduodenal infusion [8]. Over half of patients progress from L-DOPA-responsive to L-DOPA-induced dyskinesias within 5–10 years [9]. At this point there are few options for drug treatment besides careful monitoring of L-DOPA dosage. One option that is increasing in prevalence is the use of deep brain stimulation (DBS).

Even for those who are good candidates, DBS poses its own challenges. Good candidates for DBS are patients who have maintained their cognitive functions and are still responsive to L-DOPA but have disabling dyskinesias, medication-resistant tremors, and on-off fluctuations [10]. In many cases multiple passes through brain tissue are required to hit the intended target, typically the subthalamic nucleus or the internal globus pallidus. Each needle pass carries with it the risk of infection, bleeding, stroke, or other damage to the brain. The advent of intraoperative-guided DBS has addressed the multiple pass problem, but this technology is not yet available to most DBS centers. Second, DBS introduces permanent hardware into the brain, neck tissues, and chest wall, which can result in infection, breakage of the system with normal neck use, or physical trauma to the head, neck, or chest. The hardware required limits use of diagnostic MRI scans, requires special surgical precautions, and causes interference with EKGs and other possibly needed emergency diagnostics. Other limitations of DBS are that the batteries for the system need periodic replacement, and programming of the system is complicated and requires clinic visits. DBS often does not treat the most disabling symptoms of PD, such as gait imbalance, gait freezing, and speech dysfunction. If a candidate has underlying speech disturbance, DBS can aggravate this problem. The drawbacks and limitations of L-DOPA and DBS therapies highlight the need for improvement in treatments for PD. There is evidence to suggest that cell replacement therapy may offer an alternative that would provide long-lasting relief from symptoms.

History of Cell Therapy

Techniques for successful transplantation of neural tissue to the brain were reported in the 1970s [11] and first used in a Parkinson's disease model in 1979 [12, 13] (Fig. 11.1). Hemiparkinsonism can be induced in animals through unilateral injection into the *substantia nigra* with a toxin, generally 6-hydroxydopamine (6-OHDA) in rats and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) for primate and mouse studies. These models replicate several aspects of the disease and depletion of DA neurons can be indirectly tested through tests of motor behavior. Lesioned animals will rotate ipsilaterally to the lesioned side of the brain after

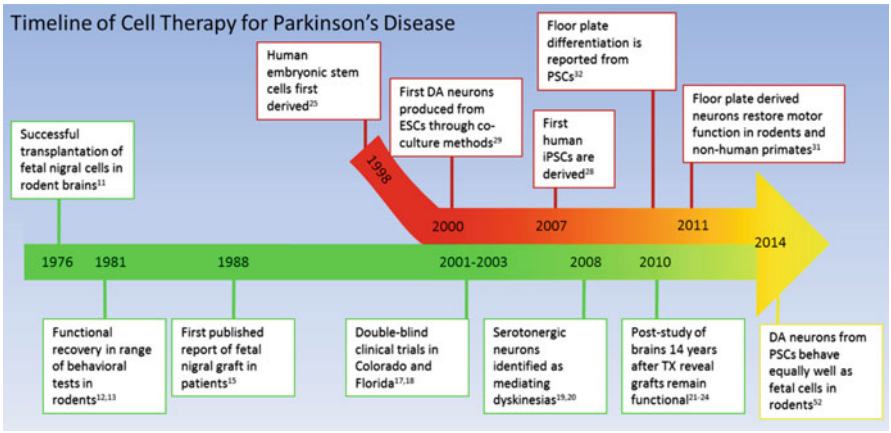

Fig. 11.1 A timeline of fetal cell therapies (*green*) and stem cell-based differentiation of DA neurons (*red*)

stimulation with amphetamine, which stimulates dopaminergic activity. Lack of dopaminergic output on the lesioned side of the brain is responsible for the unbalanced movement. This provides a useful model to investigate cell therapies because transplanted cells engraft, differentiate, and mature and are able to respond to stimulus with dopamine production, thereby reducing the imbalance in rotations. The early studies demonstrated that tissue dissected from fetal brains could engraft and reduce motor symptoms in the PD model. Interestingly, the general principle of these studies and the rodent model continue to be heavily relied upon in current studies.

Fetal Nigral Transplants

Techniques for transplanting and isolating fetal tissue improved over the next decade. Some of the first experimental treatments performed in humans used human adrenal medullary tissue [14]; not surprisingly these were unsuccessful due to the low percentage of dopaminergic neurons in the adrenal medulla, which consists of a high percentage of adrenergic neurons that further convert dopamine to norepinephrine and epinephrine. More success was had with fetal ventral mesencephalon transplants first used in the late 1980s [15]. Some patients reported dramatic improvements in motor function [16] such that two open-label, double-blind clinical investigations were performed in the United States [17, 18]. The clinical trials conducted in the United States are still a subject of controversy in part because of the study design. First, the two studies differed in their methodologies, including the length and duration of immunosuppression. In both cases, multiple fetuses were used for sourcing of the cells, with tissue from up to 6 different fetuses being used

to treat a single patient. The Colorado study was designed with an endpoint 1 year after surgery based on patient response as to whether or not their quality of life was improved. In hindsight, this study endpoint would have been improved if the primary outcome variable was based on more quantitative measurement of motor recovery and patient follow-up was extended to 2 or 3 years posttransplantation. Recent studies of the development of human DA neurons in vitro has demonstrated that human neurons take a considerable amount of time to mature, which suggests that their benefit may not be apparent until they have matured and integrated within the host tissue. Future studies should take this time period into consideration and increase the time before the actual benefit or lack thereof of transplanted cells would be fully evaluated. Breakdown of the patients into age groups revealed that those under the age of 60 responded better to the treatments than patients over the age of 60, suggesting the possibility of reduced capacity to adapt and integrate new cells into the aging brain. Additionally, it was observed that greater postsurgery decreases in PD symptoms correlated with presurgical response to L-DOPA therapy. If the transplanted cells do not recapitulate the nigral-striatal pathway complete with proper excitatory and inhibitory inputs, the grafted cells may simply act as a localized delivery vehicle for dopamine. Thus, patients who respond well to dopamine replacement could be expected to respond well to cell therapy.

Severe graft-induced dyskinesias (GIDs) were a troubling side effect observed in a small, but not insignificant, percentage of treated patients. Concerns related to GIDs were such that a voluntary moratorium on fetal transplantation was in effect until the cause could be ascertained and the treatment improved. Further study of patients with GIDs led to the idea that the presence of high levels of serotonergic neurons in the grafted tissue was responsible for the dyskinesias [19]. Imaging of patient brains experiencing GIDs revealed high levels of serotonergic activity in grafted tissue and dyskinesias were dramatically attenuated when a serotonin receptor agonist was given systemically. It was hypothesized that the grafted serotonergic neurons were interacting inappropriately with the dopamine signaling network and contributing to the GIDs [20].

One of the core arguments in favor of continued research into cell therapy for PD is the wealth of knowledge that was acquired from the patients treated with fetal cells. Some patients were studied for years after transplantation and their brain analyzed post mortem. Several key issues have been studied in this population including presence of PD pathology in transplanted tissue and the lifetime and functionality that can be expected from transplanted tissue. Several studies have been published that examined the brains of patients receiving fetal nigral transplants over a decade previously [21–24]. Two of studies reported finding Lewy-body pathology in the grafted cells, but others did not. Importantly, all the studies agreed that grafted neurons were still alive and functional as indicated by PET brain imaging prior to death. This raises an important question for PD cell therapies: Is the Parkinson's disease brain a hostile environment for transplanted tissue that will lead to neuronal death or eventual loss of function? Furthermore, would the use of autologous tissue increase the chances or the rate of disease progression in grafted tissue?

In retrospect, the fetal studies produced mixed results and revealed the potential for side effects. The inconsistencies and side effects observed in the two American studies highlight both the drawbacks of the use of fetal tissue and the lessons that can guide future approaches. The cases in which transplants resulted in dramatic and long-lasting improvement provide proof of concept that cell therapy can be used to help at least a certain population of PD sufferers and serve as a tantalizing goal for all future cell therapies for neurological diseases.

Stem Cell Differentiation of DA Neurons

The scarcity of high-quality tissue, donor variability, and dependence on skillful dissection are all disadvantages of fetal tissue that could potentially be addressed through the use of pluripotent stem cells (PSCs). PSCs can now be derived from several sources, including the inner cell mass of a fertilized embryo [25], parthenogenetic embryo [26], or an embryo created by somatic cell nuclear transfer [27] (human embryonic stem cells; hESCs), or through introduction of exogenous transcription factors to adult somatic cells [28] (induced pluripotent stem cells; iPSCs). Each cell source has different advantages and disadvantages including autologous vs. allogeneic sourcing. PSCs can be grown in large quantities and differentiated into DA neurons or their progenitors and can be highly characterized through gene and protein expression analysis as well as functional testing such as electrophysiological characterization or measurement of secreted neurotransmitters. Differentiation strategies for producing DA neurons from PSCs have progressed greatly in recent years.

The first reported successes in generating dopaminergic neurons from PSCs either used stromal cell coculture with PA6 or MS-5 mouse stromal cell lines [29]. Coculture allows for differentiation signals to be passed through direct adhesion as well as paracrine secretions; however, it is difficult to probe the role of individual molecules in the differentiation process. Differentiation of PSCs to neural rosettes, which are columnar epithelial cells that grow in a radial pattern, is a much used method to generate neuronal stem cells which are capable of differentiating to various neuronal cell types [30]. Rosettes were first produced through embryoid body-based differentiation protocols using spontaneous differentiation of PSCs grown in suspension and this approach required manual isolation of the rosette cells identified by their morphology. Neural stem cells derived from rosettes can be induced to differentiate into cells that express many of the typical A9 DA neuronal markers, including tyrosine hydroxylase, PITX3 (paired-like homeodomain 3), and the inward rectifying potassium channel GIRK2. The same morphogens that are known to be responsible for patterning of the brain after the formation of the neural tube are used to direct differentiation, including sonic hedgehog (SHH) and fibroblast growth factor 8 (FGF8).

The derivation of DA neurons from stem cells was further advanced by development of protocols that allowed for production cells that mimic cells of the

mesencephalic floor plate [31, 32]. Cells of the floor plate secrete SHH and Netrin 1, which promote organization and patterning of the neural tube. Floor plate cells were generally considered to be non-neurogenic until a series of studies determined that the cells of the mesencephalic floor plate give rise to DA neurons in the *substantia nigra* [33, 34]. Timed exposure to morphogens directly at the PSC stage can produce cells that express transcription factors characteristic of floor plate cells, namely LMX1A (LIM homeobox transcription factor 1, alpha) and FOXA2 (forkhead box A2) with additional expression of OTX2 (orthodenticle homeobox 2) providing neurogenic potential. Floor plate differentiation can be achieved with high efficiency using monolayer differentiation, allowing for faster differentiation times and greater control over the cellular microenvironment. Scale-up production of cells in monolayers is more difficult than suspension culture techniques, but production of large batches of DA neurons or progenitors may not be needed for cellular treatment of PD. Analysis of brains of patients who had received beneficial fetal grafts found that approximately 100,000 surviving DA neurons could provide substantial benefit [35], which contrasts greatly with the millions or billions of cells which are thought to be needed for cell therapies for other non-neural diseases (Fig. 11.2).

Product Characteristic	TEST	Desired Outcome
No $OCT4^+$ cells	RT-qPCR for lin28 Tumorigenicity tests	Eliminate possibility of teratoma formation
Low $PAX6^+$ cells	Gene expression, protein expression	Reduce graft overgrowth
Progenitor expression of FOXA2, LMX1A, EN1 and CORIN	Gene expression, protein expression analysis	Authentic midbrain DA neurons
In vivo expression of GIRK2, PITX3, TH, DAT, VMAT but not Serotonin	Histological analysis of graft or prolonged in vitro maturation	Authentic midbrain DA neurons
Autologous vs. Allogeneic		Autologous cells may engraft at higher efficiency

Fig. 11.2 Desired characteristics of cells used for cell therapy and the methods in which these characteristics are analyzed

The increased use of small molecules and library screens based on phenotypic characterization have given stem cell biologists greater control over cellular differentiation [36]. Small molecules are robust and relatively cheap to produce compared to growth factors allowing for large phenotypic screens to be performed routinely. For example, floor plate tissue can be efficiently induced through the timed exposure to small molecules LDN193189 and SB431542, which inhibit both SMAD signaling pathways. SMAD signaling in early stem cell differentiation is driven by bone morphogenetic proteins and activin, which promote mesendoderm differentiation. By blocking SMAD signaling, ectoderm differentiation is enhanced in PSCs [37]. The small molecules purmorphamine and CHIR99021 can strongly activate SHH and WNT signaling, respectively, and are used in combination with FGF8 and SHH to induce floor plate differentiation.

Several groups have adopted a floor plate-based differentiation strategy because of the ease of producing floor plate cells and, more importantly, convincing in vivo data directly comparing floor plate-derived DA neurons and rosette-derived DA neurons. In this study [31] better and longer lasting functional recovery was observed when floor plate-DA neurons were used. Floor plate grafts had higher levels of dopaminergic gene expression and lower levels of serotonergic and GABAergic neurons and maintained recovery in amphetamine rotations through 16 weeks posttransplantation, while rodents given rosette-derived grafts showed brief recovery before deteriorating to pre-transplantation levels. While definite conclusions cannot yet be made, the floor plate protocol uses a rational, biologically inspired approach and likely produces more authentic A9 DA neurons than rosette-based differentiation.

Even with robust differentiation protocols, the timing of transplantation can be important with regard to cell specificity and engraftment. In one study, genetically modified cells were used to mark progenitors, early DA neurons, and mature DA neurons and each population was transplanted into a rodent model of PD [38]. The study demonstrated that early progenitors engrafted better than mature neurons; however, there was a lower percentage of DA neurons in the grafted cells. The authors posited that cells expressing NURR1, a nuclear receptor that induces expression of tyrosine hydroxylase, but not PITX3, a later marker of differentiation, were optimal for transplantation because of high engraftment efficiency and high DA specificity. In some cases, longer differentiation can be required in order to reduce the possibility of residual undifferentiated PSCs remaining in the population [39]. The differences between methods likely stem from the use of embryoid bodies (EBs) in some protocols, in which the differentiation is more difficult to control and pockets of pluripotency can remain for long periods of time through the development of microenvironments within the EB which are permissive of self-renewal [40]. Several groups have used fluorescence-activated cell sorting to enrich for DA neurons either during the differentiation process or prior to transplantation [41, 42]. This has several advantages, including the elimination of residual undifferentiated cells. Highly sensitive tests have been developed to detect low levels of undifferentiated cells, down to 1 cell in 50,000 differentiated cells using qRT-PCR analysis of gene expression [43]. Residual PSCs are a concern because of the fear

of tumor formation in any cell therapy using cells derived from PSCs [44]. This concern has been allayed somewhat by the safe use of PSC-derived therapies for spinal cord injury (Geron trial, unpublished data) and macular degeneration [45] without any evidence of adverse events related to the transplanted cells.

A recent study directly compared DA neurons differentiated from PSCs using the floor plate protocol to human fetal nigral tissue in a preclinical rodent model [46]. The PSC-derived cells performed as well as fetal nigral tissue, resulting in long-term motor recovery of at least 6 months in rodents, thereby providing more evidence that PSCs can be differentiated into authentic midbrain DA neurons. PSC-derived transplants innervated striatal and extrastriatal midbrain DA targets and exhibited equal axonal growth when compared to fetal cells. It is important to emphasize that establishment of equivalency of potency between PSC-derived cells and fetal cells is critical to the argument that stem cell-derived therapies could potentially match the successes previously observed while at the same time providing batch-to-batch consistency and quality control measures needed to address the drawbacks of using fetal cells.

Understanding of the mechanism of action is helpful to both design clinical trials and for support for application for clinical approval to the US Food and Drug Administration (FDA), which controls approval to perform clinical trials. The mechanism of action of cell transplants has long been thought to be increased dopamine signaling, but more recent studies have been able to further investigate intricacies of the interaction between the transplanted cells and the host tissue. For example, cells genetically engineered to express light-sensitive proteins, a technique called optogenetics, were used to investigate the mechanism of functional recovery of transplanted floor plate-derived cells [47]. By using light to disable the effects of the grafted tissue, the study demonstrated that functional motor recovery was due to direct activity of grafted cells; when transplanted cells were deactivated by light, the rodents again displayed motor dysfunction and preferential movement ipsilateral to the lesioned area of the brain. Further ex vivo study of slices of rodent brain determined that grafted tissue was interacting with host neuronal tissue through the formation of neuronal networks similar to endogenous brain networks. Stimulation of glutamatergic neurons in the corpus callosum resulted in increased dopamine secretion and stimulation of medium spiny neurons downstream from the transplanted DA neurons, an effect that was observed in grafted rodents but not in rodents with lesion only.

The development of authentic midbrain DA neurons in vitro has largely coincided with the development of induced pluripotent stem cells (iPSCs), cells produced by introduction of exogenous factors capable of reprogramming somatic cells into PSCs that are essentially identical to embryonic stem cells [28] (Fig. 11.1). iPSCs derived from PD patients are another potential source for cell therapy and can also be used for modeling PD in a dish using human cells, an important tool for complex neurological diseases like PD which are difficult to fully capture in animal models. iPSCs can be created from PD patients and then genome editing techniques can be used to study the effects of PD-associated mutations on cell behavior [48]. DA neurons differentiated from patient iPSCs

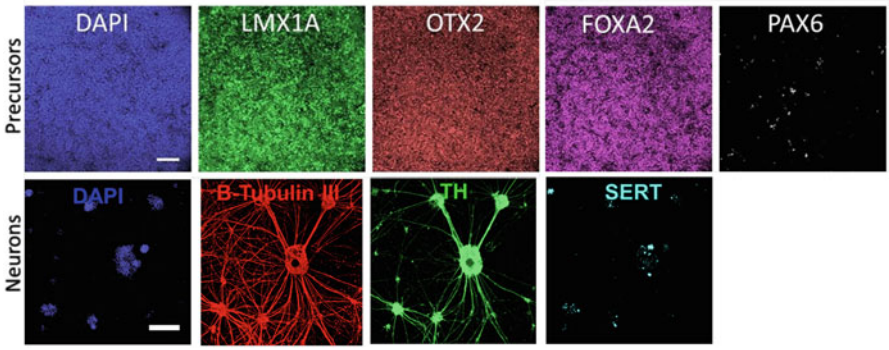

Fig. 11.3 Representative iPSC differentiation toward floor plate precursors expressing FOXA2, OTX2, and LMX1A but low levels of PAX6. Neurons differentiated from these progenitors are largely TH positive but do not express serotonin

with triplication of the *SNCA* gene, which encodes for α-synuclein, produced double the amount of α-synuclein protein as compared to neurons from an unaffected relative [49]. There is a limitation to modeling of late-onset diseases, such as PD, using iPSCs. The reprogramming process erases age-related markers and increases telomere length [50], but because PD is not a developmental disorder, iPSCs from PD patients produce healthy neurons at the same efficiency as unaffected stem cell lines (Fig. 11.3). On the positive side, this means that PD patients are excellent sources of autologous DA neurons.

Current Approaches to Stem Cell Therapy

After extensive animal testing, cell therapies are being planned by several groups for human experimentation. Cell therapy can be implemented using autologous, allogeneic, or even xenogeneic cells. While fetal pig neurons were actually used in patients in the 1990s [51], current proposals for cell therapy focus on using human ESC or iPSCs. The advantages of allogeneic therapy are that only a single hESC or iPSC line is required, the differentiation protocol only has to be perfected for one cell line, and large batches of DA neurons could be made and quality tested before use. The PSC-derived DA neurons could be frozen and used "off the shelf" to treat large groups of patients. In contrast, for autologous therapy, iPSCs must be made for each patient, greatly increasing the time and resources required to prepare cells for transplantation. The advantages of autologous therapy, however, may overcome the disadvantages. Most importantly, patients transplanted with their own iPSC-derived cells should not require immune suppression; in a recent study in nonhuman primates, DA neurons from autologous iPSCs showed better survival and outgrowth than those from an allogeneic donor [52].

While the US FDA has approved clinical trials for allogeneic hESC-derived products for macular degeneration [53] and spinal cord injury [54], there are no established pathways in the United States to regulatory clearance for iPSC-based therapy. However, in Japan, which is a leader in the study and in use of iPSCs, the first patient was treated for macular degeneration with cells derived from her own iPSCs [55]. The most expensive component of regulatory approval for a cell line in the United States is the requirement for extensive animal trials. Even with the cost of immunosuppression eliminated, if a complete set of animal studies is required for each iPSC line, the autologous therapies would be far more expensive than allogeneic approaches. An alternative to additional animal studies may be regulatory acceptance of the protocol or techniques used to obtain the differentiated cells, with regulated thresholds for quality assurance at various stages of differentiation. Molecular diagnostic tests could be used in place of animal studies to precisely determine the identity and quality of the cells. Such a test is already widely used for the identification of novel pluripotent stem cell lines based on genome-wide gene expression analysis [56]. While autologous iPSC-based therapies are currently a more costly and time-consuming option compared to allogeneic therapies, reprogramming is still an area of intense study and new improvements are expected to reduce costs and time required to produce novel iPSC lines.

Advances in cell delivery technologies have also changed the design of proposed therapies. Curt Freed described the first surgery in his studies which used 16 needle passes into the brain in order to deliver cells to a large enough area in the putamen [57]. Each needle pass carries with it a risk of hemorrhage and probable axonal damage increasing the risk and the recovery time required for the surgery. New cell delivery systems have advanced far beyond a simple needle and are capable of delivering cells at multiple depths and radial distances from a single brain penetration tract [58]. Cadaver studies and pig models have demonstrated that cells delivered in this manner can cover a large volume of a human putamen, hopefully reducing the needle passes to one per side of treatment.

Conclusions and Future Directions

There is no proven method to arrest or even slow the progressive neurodegeneration associated with Parkinson's disease. At the time of diagnosis, it is estimated that over 50 % of the DA neurons in the *substantia nigra* have already undergone cell death. Thus, until PD can be diagnosed before significant neuronal death occurs, and early diagnosis can be combined with a treatment to arrest future neuronal deterioration, cell replacement therapy deserves careful clinical study to determine its potential benefits. Experimental stem cell-based therapies should strive to reach a high bar and careful experimental design is required to ensure that clinical trials build our knowledge of regenerative medicine. Parkinson's disease is well suited for cell therapy because of the specificity of the cell type that is thought to be needed for treatment and the wealth of knowledge gained from fetal studies performed

previously. The use of pluripotent stem cells can address many of the drawbacks discovered in the use of fetal tissue and recent advances in differentiation protocols now produce what are considered to be authentic midbrain dopaminergic neurons, which perform well in several animal models of PD including nonhuman primates. At a minimum, cell therapy aims to localize delivery of dopamine to the proper region of the brain and deliver the neurotransmitter in a more stable fashion than the pulses of that are present when L-DOPA is delivered systemically. More recent studies of PSC-derived cell transplants in animal models suggest that the cells can do much more, including innervation of striatal and extrastriatal dopamine targets and recreation of proper neural networks.

Several strategies are currently being pursued around the world including the use of autologous DA neurons differentiated from iPSCs. While immune rejection is theoretically eliminated when using these cells, the effect of highly penetrant mutations may preclude some patients from this approach. The European consortium TRANSEURO plans to reboot the fetal cell studies, this time using advances in cell technologies to avoid pitfalls of previous studies [59]. Beyond the cell source, questions remain in the use of immunosuppression, cell purity, and the delivery method of the cells. However, many of these questions cannot be adequately addressed in animal models and will require careful clinical experimentation. Due to the strong placebo effect which is known to occur in PD, clinical study of cell therapy will likely require long-term follow-up and progress more slowly than other stem cell therapies, such as those for heart disease, diabetes, and macular degeneration. The success enjoyed by some of the individuals treated with fetal cells remains a tantalizing reminder of the potential for cell therapy to revolutionize the treatment of neurological disorders and it is likely that clinical trials striving to achieve this goal will be initiated within the next few years.

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Chapter 12
Could Stem Cells Be Used to Treat or Model Alzheimer's Disease?

Edsel M. Abud and Mathew Blurton-Jones

Abstract Alzheimer's disease (AD) is the leading cause of age-related dementia, affecting more than five million people in the United States alone. Unfortunately, the incidence of AD is expected to double in the next 20 years. Yet, currently approved therapies provide only marginal short-term benefit. Thus, there is a critical need to accelerate translational research for AD. One area of study that has received increasing attention is the potential application of stem cells for AD. Many studies have now shown promising preclinical benefits of stem cell transplantation in animal models. In addition, rapid progress in the development of induced pluripotent stem cells (iPSCs) is providing an exciting new platform for disease modeling and the development of future therapies. In this chapter, we will review the current state of research on stem cell transplantation for AD. We will also examine the mechanisms that underlie the effects of stem cell transplantation and the considerable challenge in translating these findings toward clinical trials. Lastly, we will discuss the potential clinical and disease-modeling applications of patient-derived iPSCs for AD.

Keywords Neural stem cells • Neuroprotection • Neurotrophins • Beta-amyloid • Tau • Inflammation • Cognition • Synaptic plasticity • BDNF

Introduction

Alzheimer's disease (AD) is the most common cause of age-related dementia and leads to a progressive loss of memory and executive function. Patients suffering from AD typically first lose declarative memory, then the ability to perform basic everyday functions, and eventually can no longer take care of themselves, robbing them of their independence. The rapid growth in the prevalence of AD arguably represents one of the greatest threats to public health in the world. In the USA alone, AD currently affects more than 5.2 million people, and every 68 s a new person is

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diagnosed with AD [1]. As world demographics shift toward an increasingly aged population, the incidence of AD is predicted to rise exponentially, such that by 2050 a new person will be diagnosed with AD every 33 s and 16 million people will be affected. Without effective disease-modifying treatments, the costs associated with caring for these patients will likely exceed $1.1 trillion per year [1, 2]. Thus, there is a pressing need to identify new and effective treatments for AD.

AD Neuropathology and the Amyloid Cascade Hypothesis

Researchers have known since Alois Alzheimer's first report in 1907 that AD patients exhibit widespread brain atrophy and two hallmark lesions [3]. Alzheimer described one of these lesions as a "special substance in the cortex" that was refractory to histological stains. Several decades later this "special substance" was identified by George Glenner and colleagues as beta-amyloid (Aβ), a small 40–42 amino acid peptide that accumulates as insoluble extracellular amyloid plaques [4]. The second hallmark pathology described by Alzheimer appeared as a "tangle of fibrils indicat[ing] the place where a neuron was previously located." Neurofibrillary tangles (NFTs) were subsequently shown by several groups to be composed of insoluble hyperphosphorylated aggregates of the microtubule-binding protein tau [5–9]. Both Aβ and tau pathologies have continued since their discovery to be a major focus of AD research and lead therapeutic targets. Yet compounds that alter the production or degradation of Aβ and the aggregation of tau have thus far failed in late stage clinical trials that attempt to treat AD after disease onset [10, 11].

Despite these failures, the "amyloid cascade hypothesis" remains the prevailing theory regarding the cause and consequences of AD. This hypothesis postulates that overproduction and/or compromised clearance of Aβ are the driving forces in AD pathogenesis that in turn lead to downstream insults including tau hyperphosphorylation, neuroinflammation, and synaptic and neuronal loss [12]. The amyloid cascade hypothesis is strongly supported by genetic cases of dominantly inherited familial AD (fAD). Although fAD constitutes only 1–3 % of all AD, these cases always involve either mutations or triplication of the amyloid precursor protein (APP) gene or mutations in presenilin-1 (PS-1) or presenilin-2 (PS-2). APP, as the name implies, is the precursor protein from which Aβ is proteolytically derived, whereas PS-1 and PS-2 make up the catalytic component of the gamma-secretase complex that releases beta-amyloid from APP. Each of these familial mutations lead to either an overall increase in Aβ production or a shift in the ratio of more aggregation-prone 42 amino acid Aβ versus the 40 amino acid form. In contrast, mutations in tau do not lead to AD but rather cause frontotemporal dementia [13]. Together, these genetic findings strongly implicate beta-amyloid as the driving factor in AD pathogenesis. As a result, many researchers and the pharmaceutical industry have focused their efforts nearly exclusively on developing therapies aimed at reducing Aβ production or increasing its clearance from the brain. It is only in the last few years following the many failures of Aβ-targeting therapies in established

disease, and the development of new imaging modalities, that the field has begun to realize that Aβ accumulates for some 10–20 years prior to cognitive dysfunction and thus better represents a preventive target rather than a therapeutic one [14]. How then can we hope to treat a disease that manifests over several decades? One area of research that aims to change this devastating prognosis is regenerative medicine and the potential application of stem cells for AD.

Properties and Sources of Neural Stem Cells

Over the last two decades, stem cells have rapidly risen to represent one of the forefronts of preclinical biomedical research. Many researchers have begun to focus on these cells because of two key properties. By definition, a stem cell is capable of self-renewal, i.e., able to make perfect copies of itself over and over again. In addition, stem cells can differentiate, changing their phenotype to become mature organ-specific specialized cells. These properties together allow researchers to not only greatly expand and study stem cell populations *in vitro*, but to also drive them toward a specific phenotype relevant to a given organ or disease. For example, neuroscientists have focused their efforts primarily on Neural Stem Cells (NSCs), multipotent progenitors that can self-renew and differentiate into the three principle cell types of the brain: neurons, astrocytes, and oligodendrocytes [15, 16]. NSCs can also be differentiated toward glial restricted progenitors such as oligodendrocyte progenitors (OPCs), allowing researchers to use a specific precursor population to study or treat disorders that effect a given cell population [16–19].

NSCs can be derived from a variety of sources including adult and fetal brain tissue or pluripotent stem cells including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Interestingly, the specific source of a given NSC population appears to dramatically influence both its properties and its potential clinical application. Fetal-derived NSCs (f-NSCs), for example, are dissociated from fetal tissue but can be highly variable, being influenced by both the developmental age of the tissue and the region from which they are isolated [20, 21]. f-NSCs derived from later developmental ages tend to mimic a more gliogenic differentiation potential, whereas f-NSCs derived from earlier gestational ages produce more neurogenic NSCs. The potential clinical application of f-NSCs is complicated not only by their variability but via two additional factors. First, f-NSCs are arguably one of the more controversial sources of NSCs as they are derived from aborted material and thus their use is tied into the highly contentious debate about abortion rights and when human life begins [22]. A second major issue surrounding the potential clinical application of f-NSCs is their limited capacity for self-renewal and expansion. Human f-NSCs can typically only be passaged 10–20 times before their differentiation potential changes and/or karyotypic abnormalities arise [23]. Each NSC line intended for clinical use needs to be generated and maintained under good manufacturing practice (GMP) conditions and should be highly tested and validated. f-NSCs therefore represent a fairly limited and expensive source of cells for

treating the growing cases of AD or other neurodegenerative diseases. Despite these important caveats, transplantation of f-NSC has led to promising results in several preclinical experiments [24, 25].

To address the problems of scale and consistency, researchers have over the past 15 years begun to examine the use of ESC-derived NSCs to both study and treat AD. As a result, huge progress has been made in the ability to culture, expand, and differentiate human ESCs [26–29]. ESC-derived NSCs provide the added benefit of scalability and decreased genetic variability between samples [30, 31]. Furthermore, ESC-derived NSCs can be genetically modified to stably express additional therapeutic genes for their use in specific diseases [17, 32, 33]. Still, the source of ESC–NSCs are not without controversy, and the use of both ESC-derived and f-NSCs may be limited by immune compatibility between donor and recipient [34–39]. In contrast, iPSCs and NSCs derived from them, iPSC-derived NSCs, provide a new approach to both model and treat human disease. The incredible potential of iPSCs to treat AD will be discussed in greater detail later in this chapter, but first we will review the current and more substantial set of studies examining the transplantation of fetal- and ESC-derived NSCs for AD.

Transplantation of Stem Cells for AD

The application of NSCs to AD research can be broadly divided into two primary areas of focus. One approach uses animal models of AD to examine the potential effects of NSC transplantation on AD pathogenesis and cognitive impairments (Figs. 12.1, 12.2, 12.3, and 12.4). The second major focus examines NSCs *in vitro* to study questions about the causes and development of AD as well as to screen and identify novel experimental therapies in human brain cells.

The methods and technologies that allow researchers to derive, propagate, and differentiate stem cells have advanced greatly over the past decade [41–45]. As a result, many studies have examined the therapeutic potential of stem cells for virtually every major human disease. Yet, despite their prevalence, studies of stem cell transplantation for AD have until recently lagged behind. Why? In large part, the answer may lie in the fairly limited way in which the field originally viewed stem cell therapies. Initially, transplantation studies for brain disorders focused exclusively on replacing damaged or lost neurons, an approach commonly referred to as cell-replacement therapy. However, studies that more carefully examined the mechanisms involved in NSC-mediated improvements (summarized in Fig. 12.1) soon revealed that NSC transplantation often influenced behavior or pathology via more indirect mechanisms, such as neurotrophin secretion or immune modulation [46, 47]. Furthermore, several studies also revealed that differentiation of NSCs into supporting glial cells might play a critical role in functional recovery [48–50]. The role and utility of replacing diseased neurons for AD therefore remain unclear. Yet, it is nevertheless important to examine the current findings and challenges of cell replacement paradigms for AD.

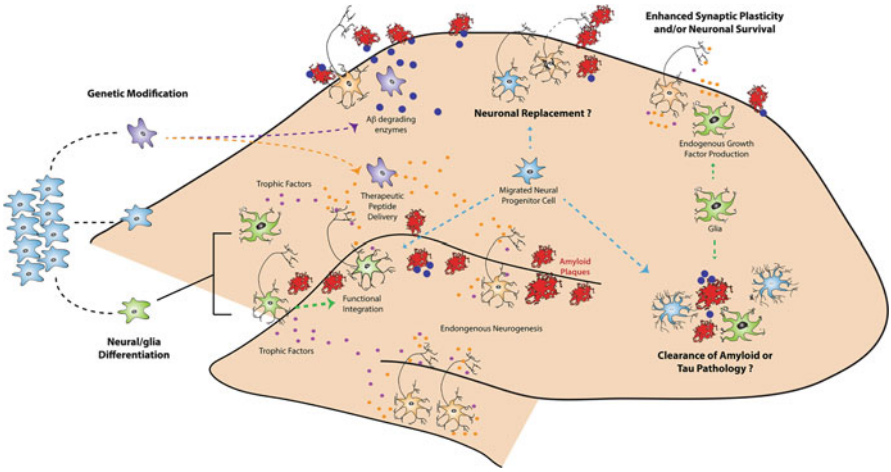

Fig. 12.1 Preclinical studies suggest that Neural Stem Cells could influence cognition and AD pathogenesis via several mechanisms. NSCs are multipotent progenitors that when transplanted in the brain (hippocampus shown) can differentiate into neurons (*blue*) or glia (*green*). These cells can in turn provide therapeutic benefits by a variety of mechanisms. Some studies have for example shown that transplanted NSC increase the synaptic connectivity of endogenous neurons (beige cells) or can promote endogenous neurogenesis. Other studies have suggested that NSCs can differentiate into appropriate neuronal subtypes and integrate into local circuitry. Yet other research has shown that NSCs can be genetically modified (*purple* cells) to deliver therapeutic proteins such as Aβ-degrading enzymes (*blue circles*) to facilitate the clearance of amyloid plaques (red)

Cell Replacement

Cell replacement therapies have long been proposed as a potential treatment for many neurological diseases in which neuronal dysfunction and death play a critical role. Given the complexity of replacing even a single neuronal population in the adult nervous system, it follows that most of those efforts have focused on diseases that primarily affect a single neuronal cell type. The greatest progress in the development of cell replacement strategies has undoubtedly been made for Parkinson's disease (PD), which is primarily driven by the degeneration of nigrostriatal dopaminergic neurons. For a thorough and insightful discussion of the many successes and challenges of stem cell transplantation for PD, please see Chap. 11. In stark contrast to PD, many different neuronal populations and multiple brain regions are affected in AD, thereby multiplying the complexity and challenges of a neuronal replacement paradigm. Yet, several groups have pursued studies of neuronal replacement for AD by focusing on one of the systems most affected in this disease, the cholinergic basal forebrain.

The cholinergic neurons of the basal forebrain were the first neuronal population to be strongly implicated in the pathogenesis of AD [51, 52]. These neurons project to the hippocampus, neocortex, and amygdala, all areas that are dramati-

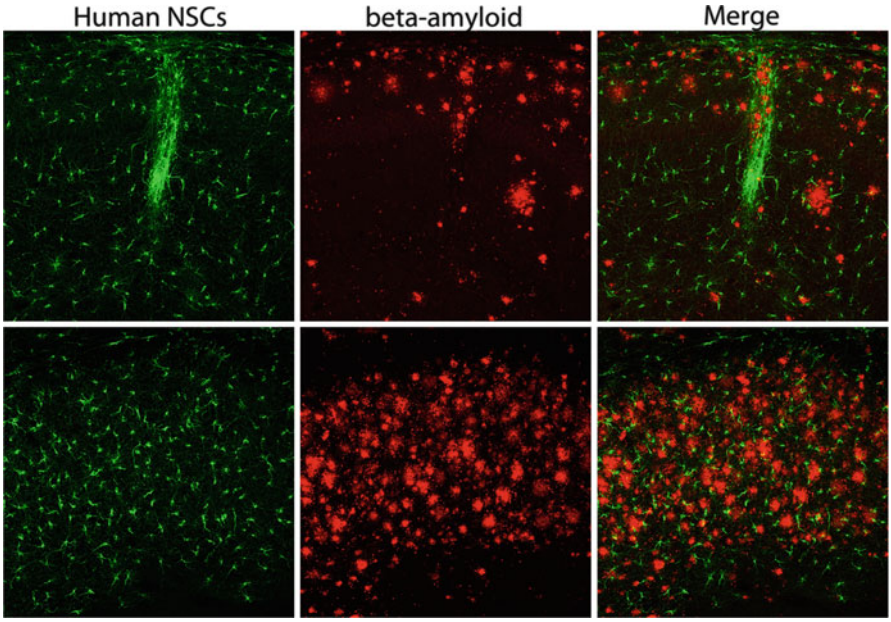

Fig. 12.2 Human NSCs can survive for over 6 months in appropriately immunosuppressed transgenic mouse models. Human NSCs (*green*) can be directly injected into the adult hippocampus via stereotactic neurosurgery. Depending on the cell source (fetal NSCs shown here), these cells can migrate extensively and appear to show little toxicity in response to extensive beta-amyloid plaque pathology (*red*). The *top panels* show a coronal section through the hippocampus near to the initial injection site (cluster of green cells). Two millimeters caudal to this injection site, the cells have migrated to the subiculum, an area of the brain with extensive plaque pathology

Fig. 12.3 (continued) Four weeks later, learning and memory were tested. (**e**) MWM training revealed that all groups learn the task. However, NSC-injected 3xTg-AD mice exhibit significantly shorter escape latencies on days 4–6 of training versus vehicle-injected transgenics (ANOVA, $p<0.04$, FPLSD $p<0.029$). (**f**) In probe trial testing, NSC-injected 3xTg-AD mice also achieve significantly shorter latencies than vehicle-injected 3xTg-AD mice and perform equivalent to NonTg controls (ANOVA, $p=0.042$, FPLSD, $p=0.010$). (**g**) Likewise, NSC-injected 3xTg-AD mice cross the former platform location more often than control-injected transgenics (ANOVA, $p=0.014$, FPLSD, $p=0.002$). (**h**) Context-dependent object recognition testing reveals that vehicle-injected 3xTg-AD mice are impaired, spending an equivalent amount of time exploring both objects. In contrast, NSC-injected 3xTg-AD mice exhibit a partial but significant recovery in this task (ANOVA, $p=0.0047$, FPLSD, $p=0.041$, vs. vehicle-injected 3xTg-AD mice). Data presented as mean ± SEM. Scale Bar = 45 μm. Figure reproduced from Blurton-Jones et al., PNAS, 2009 with authors' permission

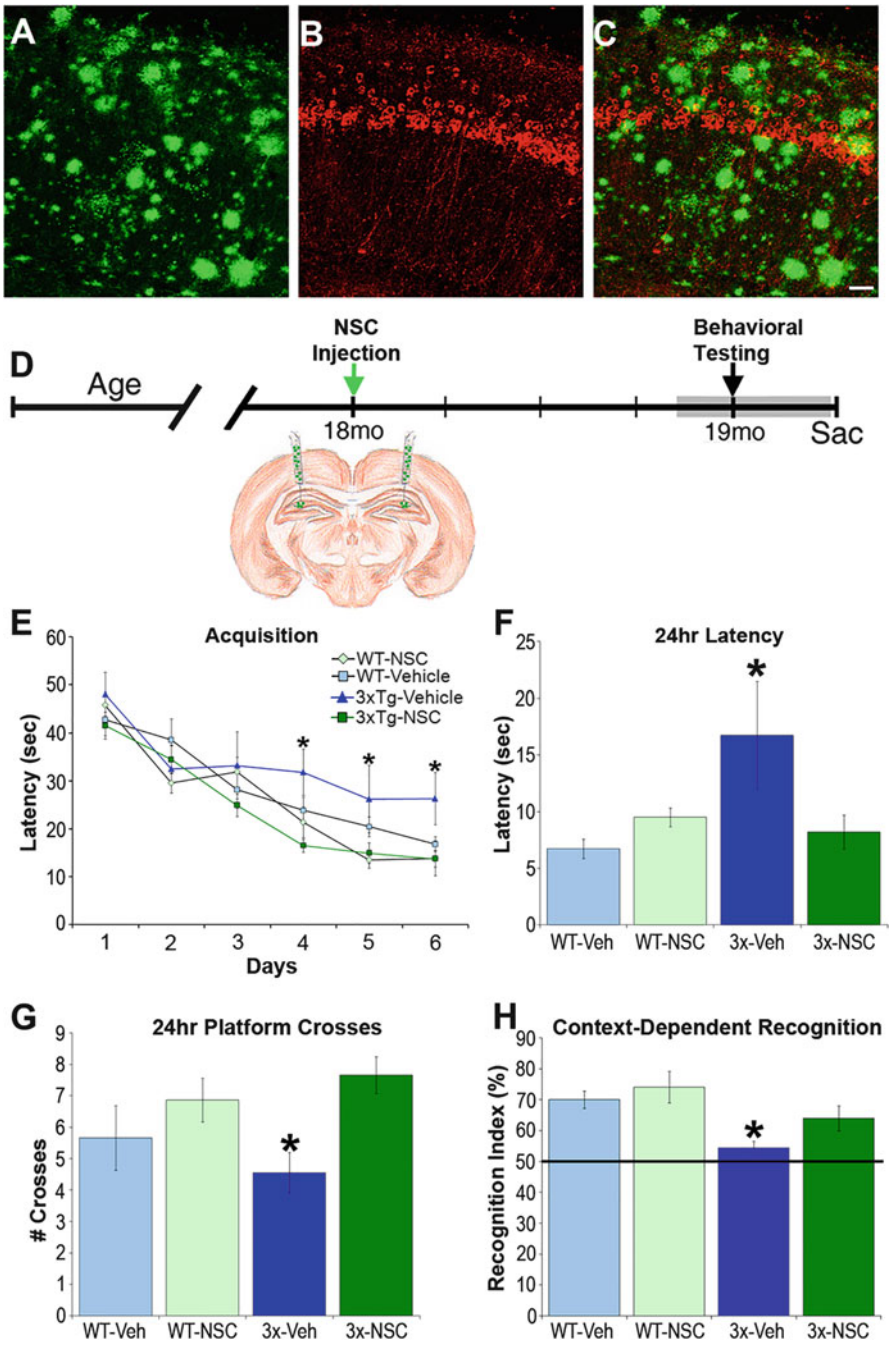

Fig. 12.3 Neural stem cell transplantation improves AD-related cognitive dysfunction. 18-month old 3xTg-AD mice exhibit robust plaques (**a**, **c**; *green*) and tangles (**b**; *red*) within the hippocampus. (**d**) 100,000 GFP-NSCs or vehicle control were stereotactically injected.

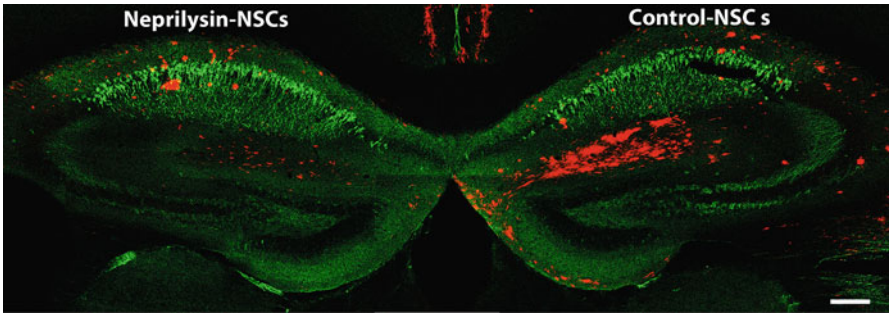

Fig. 12.4 Genetically modified NSCs decrease Aβ plaques within the ipsilateral hippocampus of 3xTgAD mice. Aged 3xTg-AD mice received unilateral transplants of NSCs genetically modified to produce Aβ degrading enzyme neprilysin on one side of the hippocampus (*left*) and control-modified NSCs on the opposite side (*right*). Three months later, sNEP-expressing NSCs had dramatically reduced Aβ plaques (*red*, OC antibody) on the ipsilateral side of the hippocampus in comparison to the contralateral hippocampus transplanted with control NSCs. Interestingly, no obvious reduction in tau (green, HT-7 antibody) was observed in aged animals, in line with prior findings that well-established insoluble NFTs are not decreased by Aβ-immunotherapy [40]. Scale Bar = 160 μm. Figure adapted from Blurton-Jones et al., Stem Cell Research and Therapy, 2014 with authors' permission

cally involved in AD pathogenesis and cognitive function. These neurons also degenerate relatively early in the disease course, and their loss correlates well with cognitive impairment [53–55]. Thus, researchers have attempted to use NSCs to replace lost cholinergic innervation in animal models. One such model uses ibotenic acid to lesion cholinergic neurons within the nucleus basalis of Meynert (NBM). This type of lesion leads to a dramatic loss of cholinergic neurons and their projections to the cortex and a resulting impairment in memory. In one study, murine ESC-derived GFP expressing NSCs were transplanted into the prefrontal cortex of mice four weeks after a cholinergic basal forebrain lesion [56]. Eight weeks later, cognitive function was examined using an 8-arm radial maze. The researchers reported a significant reduction in the number of errors in response to NSC engraftment that restored performance to levels of sham lesioned mice. Next, they used immunohistochemical approaches to examine the engraftment and differentiation of transplanted NSCs. Although the NSCs had not been pre-differentiated toward a cholinergic fate, the authors found evidence that some of the transplanted cells co-expressed choline acetyltransferase, the enzyme that produces acetylcholine and a marker of cholinergic neurons. They therefore suggested that the NSC grafts had restored cognitive function by providing a replacement source of acetylcholine to the deafferented cortical neurons. This study provides an example of how cell replacement might provide at least some benefits not by recapitulating the normal host circuitry, but rather by chronically secreting a missing neurotransmitter. This approach parallels that used in both

preclinical and clinical trials for PD, in which dopaminergic precursors are typically transplanted into the striatum, the efferent target of the lost substantia nigra neurons rather than the substantia nigra itself. In a similar study, another group transplanted hippocampal-derived NCSs in to rats that had been subjected to a fimbria fornix transection [57]. This model again produces degeneration of basal forebrain cholinergic neurons, although in this case the projections to the hippocampus are primarily affected. Following the lesion, rats received transplants of strain-matched NSCs and NSC-derived glial cells that had been pre-labeled with BrdU to facilitate identification. Just four days later, rats were tested in a Y-Maze spontaneous alternation task that measures aspects of short-term spatial memory. The authors reported that transplanted NSCs proliferated (increased BrdU labeling) and surprisingly within just a few days suggested that the cells had differentiated toward a cholinergic fate as evidenced by co-labeling of BrdU with p75, the low-affinity pan-neurotrophin receptor, a commonly used marker of choloinergic neurons. Interestingly, the engraftment of NSCs also correlated with improved cognitive function whereas NSC-derived glia provided no benefits. At face value, this study again suggests that replacing cholinergic neurons could be beneficial for AD. Yet, several problems with the interpretation of this study make such an assertion less convincing. For example, BrdU has been shown to readily leak from engrafted cells and, thus, uptake from damaged endogenous cholinergic neurons undergoing DNA repair could lead to labeling. It also seems unlikely that transplanted NSCs could differentiate into cholinergic neurons within just 4 days; rather these results may simply reveal that NSCs express P75, a finding that was recently confirmed [58]. Although these initial studies suggest that delivery of cholinergic precursors might provide meaningful benefit, they clearly also suggest that a great deal of more research is needed to move neuronal replacement strategies forward as a possible therapy for AD.

While these studies provide some intriguing results, they fall short of identifying the underlying mechanism(s) by which NSC transplantation may influence cognition. They also do not represent attempts to fully recapitulate the endogenous circuitry. This is of course the greatest challenge of any neuronal replacement paradigm. To fully replace cholinergic neurons, a great deal of more needs to be achieved that simply transplant cells and induce cholinergic differentiation. Most challenging of all would be to find ways to coax these transplanted cells into projecting their axons great distances through the adult brain to their appropriate targets. In the case of cholinergic neurons, this would be incredibly complex as axons would need to project through white matter tracts such as the fimbria fornix or to multiple targets throughout the neocortex. These studies therefore highlight the reality that neuronal replacement is far more complex than simply transplanting neurons. Unfortunately, there are many additional issues with neuronal replacement strategies for AD. Perhaps the most significant issue is that AD affects many more neuronal systems than just the cholinergic basal forebrain [59–61]. For these reasons, most groups have instead focused their efforts on other therapeutic mechanisms and applications of stem cells for AD.

Neurotrophic Mechanisms

Over the last few years, a growing number of studies have examined the role of neurotrophins in stem cell-mediated functional recovery [35–37, 62–68]. Yet, the use of stem cells to deliver neurotrophins to the brain predates these studies by nearly two decades. In one of the first examples, Anders Björklund and colleagues modified NSCs to overexpress nerve growth factor (NGF) and then transplanted them into rats with fimbria fornix transections. Remarkably, this approach led to a complete rescue of medial septum cholinergic neurons, suggesting that NSCs could represent a strong candidate for delivering neurotrophic factors to damaged neuronal systems [69]. Following up on these findings, Björklund subsequently tested this same approach in aged rats, finding that NGF-producing NSCs could also prevent age-related cholinergic neuronal atrophy and improve cognition [70]. Other groups soon began to apply this technique to other disease models, using NSCs to deliver varying neurotrophins to varying models of neurodegeneration including PD, Huntington's disease, and amyotrophic lateral sclerosis (ALS) [71–73].

The use of NSCs to deliver neurotrophins to the brain continues to be a major research focus. However, some NSC populations appear to produce sufficient neurotrophins to provide benefit without genetic modification. For example, our own group has found that murine NSCs derived from P1 mice can produce high levels of brain-derived neurotrophic factor (BDNF) [62]. Likewise, Tuszynski and colleagues found that the murine C17.2 NSC line produces high levels of BDNF, GDNF, and NGF [74]. If equivalent human NSC populations can be identified, this could potentially diminish the regulatory hurdles to clinical testing by avoiding the need to combine genetic modification with cell transplantation. In our own study, we employed these BDNF-producing murine NSCs to examine the effect of hippocamapal transplantation in aged 3xTg-AD mice, a transgenic model that mimics many of the salient features of AD (Fig. 12.3). Interestingly, we found that NSC transplantation had no effect on the underlying Aβ and tau pathology. Instead, NSCs elevated levels of BDNF within the brain and enhanced both hippocampal synaptogenesis and cognitive function (Fig. 12.3). To further understand the role of BDNF in these effects, NSCs were modified via stable shRNA expression to reduce BDNF protein production by over 80 %. When a new cohort of transgenic mice were transplanted with these BDNF-deficient cells, both cognitive recovery and the synaptic effects of transplantation were lost, demonstrating the critical role of NSC-derived BDNF in functional recovery [62]. A year later, another group showed that murine neurospheres modified to overexpress BDNF had a similar beneficial effect in a neurotoxin lesion model and that BDNF-expressing NSCs could reduce toxin-induced apopotosis via activation of Akt/ERK pro-survival signaling [75, 76]. More recently, transplantation of embryonic murine NSCs into the hippocampi of APP/PS1 transgenic AD mice was found to improve spatial learning without altering Aβ pathology. Similar to our findings, these authors also found that these beneficial cognitive outcomes

were associated with increased expression of BDNF [77]. Thus, delivery of neurotrophins, such as BDNF via either unmodified or genetically engineered NSCs, appears to provide consistent cognitive and synaptic improvements in varying animal models of AD.

Delivery of Disease-Modifying Proteins

To date, almost all studies of NSC transplantation for AD have found that stem cells have no effect on the underlying beta-amyloid or NFT pathology [62, 77]. It therefore remains quite possible that NSCs could lose efficacy over time as disease-associated pathogenesis continues unabated. For this reason, several groups have begun to explore the use of NSCs to concurrently deliver disease-modifying proteins. Employing an ex vivo gene therapy approach, NSCs can for example be modified to produce Aβ-degrading enzymes or other proteins designed to target AD-associated pathology. Our group recently examined the effects of NSCs that were genetically modified to overexpress and secrete the Aβ-degrading enzyme, neprilysin (NEP). Three months after unilateral transplantation into the hippocampi of aged 3xTg-AD mice, we found a dramatic reduction in Aβ pathology in comparison to control-modified NSCs (Fig. 12.4) [32]. Interestingly, regions that receive efferent projections from the NEP–NSC engrafted hippocampus also showed reduced Aβ pathology; thus, stem cell-mediated neprilysin delivery could potentially provide fairly widespread disease-modifying effects. Similar results were obtained in a second AD transgenic model (Thy1-APP mice), and both experiments revealed increased synaptic density in NEP–NSC engrafted hippocampi. Taken together, these results suggest that NSCs may offer a promising approach to deliver Aβ-modifying enzymes to the brain and provide a combinatorial approach to treating both the pathology and synaptic deficits associated with AD.

Using a similar approach, another group examined the effect of NSCs that were modified to secrete Metalloproteinase 9 (MMP9) [78]. Like neprilysin, MMP9 has also previously been implicated in the degradation of Aβ [79]. NSCs were therefore modified to express and secrete MMP9 and transplanted into the brains of two AD transgenic models. Interestingly, MMP9 overexpression significantly increased the engraftment and survival of transplanted NSCs, yet had no effect on Aβ pathology [78]. However, the transplanted cells in this study were mainly localized to white matter tracts, and thus the increased levels of MMP9 may not have effectively reached plaques within the cortex or hippocampus.

A number of questions remain as to how well these kinds of approaches can be adapted for clinical use in AD. For example, could other proteins be expressed that might influence tau pathology? Given the interest in Aβ and tau antibody-based therapies, perhaps delivery of single chain antibody constructs would offer another approach worthy of examination. A major challenge with moving these kinds of combinatorial approaches to the clinic however relates to regulatory hurdles. It is currently very challenging to move a stem cell-based approach toward phase one

trials at least in part because it is such a new area of research, and FDA officials are attempting to establish and define the necessary conditions for regulatory approval of investigational new drug applications. However, if a stem cell is also genetically modified, it requires an even greater level of review and scrutiny. How the cell is modified becomes critical, for example, random integration of a transgene could prove to be deleterious. Fortunately, newer techniques have recently been developed to simplify the targeting of a transgene to a "safe-harbor" locus that does not disrupt the function of an important gene. Likely, the use of these kinds of methods will become increasingly standard and should therefore address this important safety challenge.

Modulation of Neuroinflammation

It should come as no surprise that the transplantation of allogeneic cells directly into the central nervous system induces a significant immune response (reviewed in: [80]). Even transplantation of autologous cells into the brain would be expected to elicit a response to the breakdown of the blood brain barrier and dead or dying grafted cells. Could this immune response however play a beneficial role in functional recovery? Some research is starting to suggest that this may indeed be the case. In one recent example, transplantation of human ESC-derived NSCs into a viral model of multiple sclerosis led to improved motor function. Yet, when the transplant was examined, no engrafted cells remained. Rather, the researchers found that the human cells had been rejected by the host immune system, but this process had also elicited an increase in T-regulatory cells that had in turn diminished neuroinflammation and produced functional recovery [81]. In this case, xenotransplantation led to a shift in the CNS immune response with beneficial consequences.

As AD involves significant activation of the innate immune system, researchers have begun to explore the potential role of immune modulation in stem cell-mediated functional improvements. To date, the majority of these studies have focused not on NSCs, but rather on mesenchymal stem cells (MSCs) [82]. MSCs are heterogeneous stromal support cells found in various tissues but predominately in adipose tissue, bone marrow, and umbilical cord blood. These cells are known for their rapid migration to sites of injury where they can secrete supportive peptides including some neurotrophins [83, 84]. However, most studies to date have used MSCs in an attempt to modify the immune response to AD pathology. In one example, transplantation of bone marrow-derived MSCs into the cortex of double transgenic AD mice led to a significant reduction in beta-amyloid deposition and tau phosphorylation, and also improved cognition [35–37]. To explain the reduced Aβ deposition, the authors provided data suggesting that microglia had shifted toward activation of the alternative pathway (M2 phenotype), leading to phagocytic clearance of Aβ. In a second study, the same group found that umbilical cord-derived MSC transplantation could likewise improve spatial learning and reduce beta-amyloid deposition

[85]. Again, the authors highlighted the increased number of anti-inflammatory microglia present in brains receiving MSC transplants.

Taken together these studies suggest that manipulation of the innate immune system could positively impact AD pathology and cognitive deficits. However, these experiments have also shown that MSCs do not survive long term and may therefore only offer transient therapeutic effects [86]. Immune modification via small molecule drugs might therefore be far more efficacious than transplantation of nonnative mesodermal MSCs into the brain as dying MSCs might also trigger pro-inflammatory signals that could exacerbate neurodegeneration. The potential clinical application of MSCs for AD is however most challenged by the inherent heterogeneity of these cells. Several studies have for example shown that the phenotype and efficacy of MSCs can be dramatically influenced by cell culture conditions and their tissue of origin [87, 88]. Thus, MSC transplantation into the brain may prove more useful as a tool to advance our understanding of the immune response to AD than as a clinical therapy.

Unique Challenges to Clinical Translation

As detailed above, a growing number of preclinical studies suggest that stem cell transplantation might one day provide benefits to patients with AD. However, the translation of these findings into clinical trials must be approached with an abundance of caution. Stem cells not only offer novel and unique therapeutic potential, they also provide novel and unique translational challenges.

Stem Cells Self-Renew

One of the most important properties of stem cells, their ability to self-renew, also underlies their greatest clinical risk. The potential of stem cells to divide uncontrollably in the host leading to tumor formation is a major concern that must be stringently tested before any clinical trials can proceed. The risk of tumorgenesis appears to be influenced by a number of factors including the type of stem cell being used, their differentiation state, the host environment, and the degree of host immune-suppression employed. For example, undifferentiated pluripotent stem cells can readily produce teratomas in multiple tissues including the brain [89, 90]. In contrast, NSCs appear less prone to tumorigenesis, although this may be greatly influenced by the source, the culture conditions, and the methods used to pre-differentiate these cells toward a neural lineage. For example, in our own studies we have found that f-NSCs rarely form tumors even with up to ten-month engraftment periods in immune-deficient mice (Fig. 12.2). In contrast, we have found that iPSC- and ESC-derived NSCs can readily form neuroblastomas following long-term transplantation into immune-deficient mice (data not shown). It remains unclear why such

differences in tumorogenic potential are observed between pluripotent-derived versus f-NSCs, although a logical explanation may be that the later developmental age of fetal NSCs allows them to more appropriately respond to local differentiation and migration cues within the adult brain. In support of this, we find that fetal-NSCs migrate more readily than pluripotent-derived NSCs, and other groups have shown that fetal- versus ESC-derived NSCs show considerable differences in gene expression profiles, suggesting these populations remain quite distinct [91].

There currently exists only a very small amount of data regarding the tumorigenic potential of NSC transplantation in the human CNS. In one phase I study, four young boys suffering from Pelizaeus-Merzbacher disease (PMD), a rare leukodystrophy, were transplanted with allogeneic f-NSCs [92]. The patients were immunosuppressed for 9 months and MRI and clinical data collected over a year. This small study suggested a favorable safety profile as no signs of adverse effects or tumorigenesis were observed in either clinical or MRI analysis. In stark contrast to these promising findings is a case report of a young boy suffering from ataxia telangiectasia [93]. This patient had traveled to Russia to receive human fetal NSC transplants on three separate occasions. Four years after the first transplantation, however, the patient began experiencing frequent headaches and an MRI revealed several tumors within the brainstem and spinal cord. The boy's tumors were subsequently resected and found to consist of genetically female cells, indicating that these tumors were indeed derived from the fetal NSC grafts.

There are of course several notable differences between these two contrasting reports. The case study for example involved transplantation of uncharacterized NSCs in an unregulated setting, whereas the PMD trial was approved by the FDA and used relatively well-characterized FACS-sorted NSCs. This important difference could readily explain these contrasting findings. Alternatively, the Russian boy's tumors were not detected until 4 years after the first transplantation, whereas the PMD trial and follow-up lasted just 1 year. Thus, slow-growing NSC-derived tumors could take many years to manifest either clinically or radiologically. Regardless of the potential explanation of these contrasting findings, both studies highlight the need to carefully consider and test NSCs for potential tumorigenesis. It follows that the FDA requires considerable long-term safety and toxicology testing of candidate stem cell lines in immune-deficient models before phase one trials can proceed.

The Challenges of Scale and Delivery

A second major challenge to the translation of stem cell-based therapies for AD involves questions of scale and delivery. There are limited options available to model AD in large animals and thus the great majority of preclinical AD studies have relied heavily on transgenic AD mouse models. Unfortunately, these models have thus far provided little predictive value as many promising preclinical studies have failed to translate in clinical trials [94]. There are many potential explanations for this including the lack of significant neuronal loss in most transgenic AD models

and the likelihood that these models mimic only the earliest prodromal phases of AD [95]. However, when it comes to cell-based therapies, the issues of scale becomes increasingly important and challenging. The human brain is approximately three thousand times the mass of the murine brain. In a widespread disease such as AD, delivery of therapeutic cells to appropriate and sufficient targets will likely be especially important. Will stem cells therefore need to be injected into multiple or even numerous locations? This would of course dramatically increase the risk of such therapies and likely make them infeasible. One hope is that NSCs have been shown to exhibit considerable migratory capacity and thus perhaps only a few delivery sites will be needed to achieve widespread distribution [96] (see also Chap. 15).

Could Induced Pluripotent Stem Cells Be Used to Treat or Model AD?

In 2006, Shinya Yamanaka first described the generation of iPSCs from mouse fibroblasts. By overexpressing four key transcription factor genes in murine fibroblasts, Yamanaka and his colleagues showed for the first time that a terminally differentiated cell could be "reprogrammed" into a pluripotent state, capable of giving rise to any cell type within the body [97]. Within 1 year, both Yamanaka and independently James Thompson's group had replicated this approach using human cells, giving rise to what is perhaps one of the most rapidly evolving and exciting fields of biological research [47, 98, 99].

Transplantation of iPSC-Derived Cells

The potential clinical application of iPSCs can be broadly divided into two primary areas. As progress is made in the development of stem cell transplantation-based therapies, iPSCs could in principle provide cells that are perfectly matched to the intended recipient, having been derived from the donors themselves. Indeed, the first clinical trial of this kind of approach was recently initiated in Japan to examine transplantation of autologous iPSC-derived retinal pigment epithelium for patients with age-related macular degeneration. In terms of AD, NSCs or another relevant cell population could in theory be derived from patient iPSCs and transplanted into the brain, assuming compelling preclinical and safety data was first established. This approach no doubt holds great promise but perhaps the greatest challenge to the potential therapeutic application of iPSC-derived cells is economics. Each iPSC line intended for clinical use would need to be generated, expanded, differentiated, and tested following stringent GMP protocols. This would of course come with a very large price tag that could render testing and delivering of such therapies economically unviable. While the economics of iPSC-based therapies appear challenging, this field is advancing with incredible speed, and it is likely that the process of generating and validating GMP-derived iPSCs will become streamlined and the costs reduced.

Modeling AD with iPSCs

To date far more effort has focused on the use of iPSC to model human diseases than to treat them. For example, iPSC derived from monogenetic human disorders can provide an unlimited source of human primary cells to study the consequences and potential treatments for a given genetic mutation [100]. Along these lines, recent studies have generated and examined iPSCs from patients with rare monogenetic familial forms of AD. As previously mentioned, approximately 1–3 % of AD cases are caused by dominantly inherited mutations or triplications in the genes coding for APP or presenilin-1 (PS1) and presenilin-2 (PS2). In contrast, sporadic AD appears to involve a combination of genetic and environmental influences, although twin studies suggest that the heritability may be as high as 79 % [101]. The first report detailing the generation of iPSCs from AD patients examined the effect of PS1 and PS2 mutations on beta-amyloid generation [102]. As expected, neurons derived from these lines recapitulated an important aspect of presenilin-associated fAD: increased ratios of Aβ42–Aβ40. This report also described the first application of AD iPSCs for drug testing by examining the effects of γ-secretase inhibitors on Aβ generation.

A second study also produced and examined iPSCs from fAD cases, but extended their investigation to examine iPSCs from two sporadic cases of AD [42]. Interestingly, neurons derived from one of the sporadic cases mimicked some of the findings from the fAD cases including increased Aβ generation and tau phosphorylation. In contrast, the second sporadic case appeared no different than the control lines. These data clearly suggest that the genetic contributions to sporadic AD are heterogeneous, and many potential contributing factors need to be examined. It is possible that while one sporadic case involved genetic alterations that influenced Aβ production, the other case may have involved deficits in Aβ clearance mechanisms that would need to be studied in other relevant cell types such as astrocytes and microglia. Clearly these studies are in their infancy and suggest that multiple well-characterized iPSC lines will be needed to decipher the complex polygenetic nature of sporadic AD. Nevertheless, these first few reports represent vital steps in assessing the potential of AD iPSCs to model AD and offer an exciting new use of stem cells to model this disease and screen for potential new therapies.

Conclusions

Stem cells offer an exciting new approach to study and perhaps one day treat a wide array of human diseases. In the case of AD, research has only just begun to examine the potential use of stem cells in preclinical models. Yet, the clinical investigation of stem cells for human disease is proceeding at an unprecedented pace. It is therefore critical to carefully consider not only the potential benefits of stem cell transplantation but also the considerable challenges of translating stem cell-based therapies to

the clinic. In this chapter, we have reviewed many of key findings in the application of stem cells for AD. Yet, it is clear that a great deal of more work needs to be done before such therapies could proceed to early stage trials. Among the many challenges is the need to identify an optimal stem cell source and delivery approach. Given the protracted course of AD, it will also be essential to find novel ways to predict and test the long-term safety of stem cell-based therapies. Despite these many challenges, stem cells and patient-derived iPSCs, in particular, offer a unique approach to study and treat human disease that could one day harness the regenerative potential of these fascinating cells to provide effective therapies for the leading cause of age-related neurodegeneration, Alzheimer's disease.

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Chapter 13
Stem Cells for Amyotrophic Lateral Sclerosis

Anthony Donsante, Lindsey Nicole Urquia, and Nicholas M. Boulis

Abstract Amyotrophic lateral sclerosis (ALS) is a devastating neuromuscular disorder with rapid loss of motor function and death within 5 years of diagnosis. The pathophysiology is complex, but various types of stem cells have properties that make them well suited for treating this disease. Proof-of-principle studies in rodent models of ALS have demonstrated improvements in motor function and survival, although we have yet to reach a cure. These studies have formed the basis of 10 published and 21 planned/ongoing clinical trials. These trials are primarily safety studies and have not yielded significant evidence of therapeutic efficacy to date. Importantly, while animal and in vitro studies have suggested that cells from healthy individuals are superior to cells from diseased sources, the majority of clinical trials have used autologous transplantation. This fact may account for some of the lack of therapeutic benefit observed. However, the future of stem cell therapy for ALS may not lie in the simple autologous or allogeneic transplantation of cells. Instead, the field may move toward the use of genetically modified cells, amplifying the power of these cells to treat the disease and perhaps making moot the question of allogeneic versus autologous transplantation entirely.

Keywords Amyotrophic lateral sclerosis • Stem cell therapy • Mesenchymal stem cells • Neural stem cells • Hematopoietic stem cells • Bone marrow stem cells • Olfactory ensheathing cells

Abbreviations

ALS	Amyotrophic lateral sclerosis
ALSFRS	ALS functional rating scale
ALSFRS-R	Revised ALSFRS

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BDNF	Brain-derived neurotrophic factor
BMCs	Bone marrow cells
BMT	Bone marrow transplantation
CNS	Central nervous system
CSF	Cerebrospinal fluid
fALS	Familial ALS
FGF2	Fibroblast growth factor 2
flt3	FMS-like tyrosine kinase 3
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GDNF	Glial cell line-derived neurotrophic factor
GLP1	Glucogon-like peptide 1
GLT1	Glial glutamate transport protein
HSCs	Hematopoietic stem cells
IGF-1	Insulin-like growth factor
iPS cells	Induced pluripotent stem cells
IV	Intravenous
L1CAM	L1 cell adhesion molecule
mdf	Muscle deficient
MMT	Manual muscle testing
MSCs	Mesenchymal stem cells
Ngn1	Neurogenin 1
NSCs	Neural stem cells
OECs	Olfactory ensheathing cells
PBSCs	Peripheral blood stem cells
sALS	Sporadic ALS
SCF	Stem cell factor
SOD1	Superoxide dismutase 1
UCBs	Umbilical cord blood cells
VEGF	Vascular endothelial growth factor

Introduction

Amyotrophic lateral sclerosis (ALS), better known in the USA as Lou Gherig's disease, is a rapidly progressive neuromuscular disorder. The incidence of ALS across the entire population has been estimated to be 1.6 people for every 100,000. In the USA, 5000 new cases are diagnosed each year. The disease manifests initially as muscle weakness or stiffness, eventually developing into paralysis. Respiratory failure occurs as the disease affects the motor neurons innervating the diaphragm. Life expectancy following diagnosis is 2–5 years, with approximately 20 % of patients living longer than 5 years. Only one drug, Riluzole, has been approved for the treatment of ALS, and it extends life span by only a few months. Therefore, there is a pressing need to develop more effective therapies for this disorder.

ALS is a complicated disease, divided into two forms: familial (fALS) and sporadic (sALS). fALS patients have a family history of the disease and account for 10–20 % of all cases. The majority are transmitted in a dominant fashion. There are at least nine genes known to be associated with ALS, accounting for 68 % of all fALS cases. While sALS patients lack a family history of the disease, this does not imply a lack of genetic involvement. In fact ,11 % of sALS patients have mutations in genes identified in fALS [1]. Therefore, it is not clear that there is any substantive distinction separating fALS from sALS.

Not only is ALS genetically heterogeneous, the pathophysiology is complex. A large number of potential stressors exist, including, but not limited to, an inflammatory environment in the spinal cord, glutamate-induced excitotoxicity, inadequate trophic support, mitochondrial dysfunction, cytoskeletal abnormalities, and defects in axonal transport [2]. The amount that each of these pathological mechanisms contributes to the development of ALS is not well understood.

Considering the variability in the genetics of ALS as well as the complexity of the underlying biology, it is clear that effective therapies for ALS will need to act broadly. Stem cells have the potential to tackle several aspects of the disease. They can provide trophic factor support, releasing molecules such as insulin-like growth factor 1 (IGF-1), glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF). Some stem cells can express the astrocyte glutamate transporter and regulate extracellular glutamate levels. Others can dampen the proinflammatory environment of the ALS spinal cord. Stem cells that differentiate into neurons can integrate into the patient's neurocircuitry. Given this wide array of functions, many preclinical and clinical studies have focused on the use of stem cells from a variety of sources to treat ALS. In this chapter, we will review the preclinical evidence for stem cell efficacy, discuss the current state of clinical trials, and consider how stem cells could be enhanced in future studies.

Stem Cell Types

Several types of stem cells have been considered for ALS therapies. Since ALS is a disease of motor neurons, neural stem cells (NSCs) are an obvious choice. One might hope that NSCs could differentiate and replace dead motor neurons, reinnervating muscle. However, this goal has been difficult to accomplish in vivo. Instead, these cells may differentiate into neurons, astrocytes, and oligodendrocytes and are thought to act by secreting trophic factors, dampening inflammation, and regulating glutamate levels. Many NSCs are derived from aborted fetuses, which has elicited ethical concerns from certain segments of the population.

Mesenchymal stem cells (MSCs) are another popular candidate. They have the interesting ability to home to sites of tissue damage, potentially allowing them to be infused intravenously rather than directly into the central nervous system (CNS). They are mesodermally derived and can be isolated from a variety of tissues, including

bone marrow and adipose tissue. MSCs possess anti-inflammatory properties and can express a number of trophic factors. Since they can be isolated from adult tissues, ethical concerns surrounding most NSCs are avoided. It may also be possible to use the patient's own MSCs, avoiding the need for immunosuppression. MSCs are currently defined by three criteria: growth on plastic surfaces, the presence/absence of surface antigens ($CD105^+$, $CD73^+$, $CD90^+$, $CD45^-$, $CD34^-$, $CD14/CD11b^-$, $CD79\alpha/CD19^-$, and $HLA\text{-}DR^-$), and the ability to differentiate into osteoblasts, adipocytes, and chondroblasts [3]. However, some data suggest that MSCs from different tissues are not equivalent [4]. Therefore, they may be better thought of as a mixed population of multipotent cells.

Bone marrow cells (BMCs) and umbilical cord blood cells (UCBs) have both been applied to ALS. Like the other stem cells discussed, these cells can deliver a wide variety of trophic factors. In addition, they can differentiate into microglia which modify the environment of the spinal cord. Like MSCs, BMCs have the potential benefit of being harvested from the patient, avoiding immune complications. However, age may decrease their effectiveness [5]. UBCs, on the other hand, may differentiate into a greater variety of cell types and have lower immunogenicity for allografting [6].

Olfactory ensheathing cells (OECs) may be quite unique. These cells, found in the olfactory bulb and the lamina propria, are specialized glia. In the olfactory system, they help repair damage by guiding the axons from replacement olfactory neurons through lamina propria, allowing them to reach their targets in the CNS. Given their role in the regeneration of axons, researchers have sought to harness this power to repair damaged axons in other regions of the nervous system [7].

Autologous Versus Allogenic Transplantation

For MSCs and BMCs, autologous transplantation has been seen as a desirable route to therapy for two main reasons. First, since the cells come from the patient receiving the transplant, there is no risk of rejection by the immune system, and the toxic side effects of long-term immunosuppression can be avoided. Second, in contrast to fetal-derived stem cells, autologous transplantation avoids ethical concerns surrounding abortion.

However, with the discovery of C9orf72, at least 20–30 % of all ALS patients have mutations that are disease causing. This raises the question of whether cells from these patients have much therapeutic potential. Indeed, bone marrow MSCs derived from ALS patients have reduced Oct-4 and Nonag expression, suggesting a reduced stem cell capacity, and their secretion of trophic factors is significantly reduced [8]. Expression levels of pluripotency and trophic factors are also inversely correlated with increasing ALS score [9]. In vitro testing suggests that migration defects exist [8], and transplantation into an ischemic stroke model showed substantial defects in trafficking to the site of injury [9]. Neural stem cells derived from the olfactory bulb of mutant mice provided inferior protection when compared to cells

derived from wild-type mice [10]. In addition, bone marrow transplantation from wild type, but not ALS, mice could extend life span [11–13]. A similar observation was also found using c-kit$^+$ bone marrow cells, where wild-type cells secreted VEGF and angiopoietin 2 at significantly higher levels than SOD1 cells [14]. Taken together, these results suggest that autologous transplantation, although safer, may be substantially less effective.

Animal Models

Animal models can be powerful tools to evaluate therapies before they reach the clinic. The first (and for a decade, the only) gene associated with ALS was superoxide dismutase 1 (SOD1; copper–zinc SOD) [15]. Thus, the earliest and best characterized transgenic animal models of ALS have been based on SOD1 mutations. The vast majority of studies employ mice and rats that carry multiple copies of the human SOD1 gene with a glycine to alanine substitution at amino acid position 93 (referred to as SOD1-G93A) [16]. These animals develop progressive loss of innervated neuromuscular junction and motor function, death of motor neurons, and astrogliosis in the spinal cord, similar to that of ALS patients. The severity of the disease is dependent on the number of transgene copies present. Thus, changes in copy number alter the age of onset and the length of disease duration, leading to variation between studies. A second SOD1 mouse, similar to G93A, carries multiple copies of human SOD1 with a two base pair deletion in the codon for leucine 126 (Leu126delTT), resulting in a premature stop codon. For the purposes of this chapter, "SOD1" will refer to animals carrying the G93A allele unless otherwise noted.

Two studies described here employ the muscle deficient (mdf) mouse. These mice carry a spontaneous, autosomal recessive mutation in the *Scyl1* gene that results in neuromuscular atrophy and hindlimb paralysis [17]. These mice also exhibit gait ataxia and abnormal hindlimb posture more reminiscent of spinocerebellar ataxia type diseases. Symptoms begin at 5–6 weeks of age, far more rapidly than in SOD1 mice. Given the lack of association of *Scyl1* with ALS and the differences in disease presentation, it is not clear if this mouse truly models ALS. However, we have included mdf mouse studies for the sake of completeness.

Neural Stem Cells

Evidence from Animal Studies

Parenchymal injection into the spinal cord has been the most common delivery method in animal studies of NSCs. This method delivers cells directly into the ventral horn of the spinal cord, where the motor neurons reside. However, this approach

has two potential limitations. First, it entails the risk of direct injection into a diseased spinal cord, which could aggravate the patient's condition. Second, cells may not migrate long distances from the injection site. Therefore, a large number of injections along the length of the spinal cord may be required, which may not be practical at this time.

Several studies have investigated the 566RSC neural stem cell line developed by NeuralStem, Inc. These cells were isolated from the cervical/upper thoracic spinal cord of an aborted 8-week-old fetus. The cells are immunogenic and require adequate immunosuppression to survive [18]. When delivered into immune deficient rats, most of these cells differentiate and express neuronal markers. Three percent become astrocytes [19]. Yan et al. gave two pairs of bilateral injections into the lumbar cord of presymptomatic SOD1 mice at a dose of 20,000 cells per injection. Control animals received the same number of dead cells, killed by repeated freezing and thawing. Life span was increased by about 2 weeks, and motor function was improved [18]. A similar study in SOD1 rats using four pairs of bilateral with 50,000 cells per site gave a similar result [20]. Since these two studies only targeted the lumbar cord, it was possible that delivering cells to both the cervical and lumbar cord simultaneously would be more efficacious. Four bilateral injections into the C4–C5 region of the cervical spine and eight bilateral injections into the L4–L6 segments of the lumbar spine (20,000 cells per site) were given to presymptomatic rats. The results were similar to the previous studies [21].

In a fourth study, Hefferan et al. performed 10 bilateral injections of 10,000 cells per injection into the lumbar spinal cord of presymptomatic SOD1 rats. In contrast to the three previous studies, control animals received injections of media rather than dead cells. Although motor neuron loss and astrogliosis were attenuated, there was no change in life span [19]. It is unclear why the results of this study differ from those of the previous three. While the cell dose was lower (200,000 cells vs. 400,000), the use of dead cells could also be problematic. Cells with damaged plasma membranes release a variety of proinflammatory molecules (reviewed in [22]). In the context of the ALS spinal cord, the delivery of dead cells could aggravate the disease and speed degeneration.

Teng et al. investigated the efficacy of NSCs derived from the telencephalic ventricular zone of a 13-week fetus. Presymptomatic SOD1 mice received one to four pairs of bilateral injections of 100,000–200,000 cells per injection. Life span was increased in a dose-dependent manner and motor function improved. In contrast to the studies of 566RSC cells, the majority of transplanted cells did not differentiate. In vitro the cells secreted significant amounts of nerve growth factor, BDNF, and GDNF, which might account for their efficacy [23].

Two other studies employed unique types of neural stem cells. Neurons had previously been shown to be therapeutic in SOD1 mice [24]. Corti et al. used Lewis factor X positive, $CXCR4^+$ mouse NSCs in an attempt to select for stem cells favoring neuronal differentiation over a glial fate. 10^4 cells were injected bilaterally into the lumbar cord of presymptomatic SOD1 mice. This therapy extended life span

by 23 days and substantially reduced motor neuron loss. Forty-five percent of the transplanted cells adopted a neuronal fate, while 26 % became astrocytes and 4 % became oligodendrocytes [25].

While most NSCs employed in animal studies have come from aborted fetuses, one study has investigated the possibility of using autologous cells by harvesting the olfactory bulb, a region that undergoes neurogenesis well into adulthood. This method would avoid ethical concerns raised from using fetal-derived NSCs and the need for immunosuppression. Four bilateral injections of 1000 murine neurospheres (~620,000 cells) were given to presymptomatic SOD1 mice. Life span was increased by about a month, and the loss of motor function was delayed [10]. One interesting finding to note: some transplanted cells sent out axons down the sciatic nerve and into the muscle. However, they failed to innervate the neuromuscular junctions. Understanding this final step could have implications for the development of therapies to replace lost motor neurons.

Clinical Trials of Neural Stem Cells

Our laboratory presented the first-in-human perioperative morbidity results for 15 patients who received cervical or cervical plus thoracolumbar microinjections of human fetal spinal cord-derived NSCs. A risk escalation model was applied using six groups: non-ambulatory patients receiving five unilateral lumbar injections at L2–L4 levels (Group A1, $n = 3$); non-ambulatory patients receiving 10 lumbar bilateral injections (Group A2, $n = 3$); ambulatory patients receiving five unilateral lumbar injections (Group B, $n = 3$); ambulatory patients receiving (Tables 13.1 and 13.2) 10 bilateral lumbar injections (Group C, $n = 3$); ambulatory patients receiving five unilateral cervical injections (Group D, $n = 3$); and Group C patients receiving five unilateral cervical injections at a second time point (Group E, $n = 3$). Ambulatory patients were evaluated monthly for 3 months before surgery to generate a slope of disease progression for comparison [31].

Each injection delivered 10^5 NSCs via a patient-mounted surgical apparatus described previously (Fig. 13.1) [40]. The immunosuppressant regimen was consistent with the current standard of care for solid organ transplant. No evidence of acceleration of disease progression due to intervention was observed, and no improvement in disease progression was evident for Groups B, C, and D. Comparison of postsurgical data to predicted disease progression from preclinical observation suggested that Group E patients experienced a transient improvement in their revised ALS Functional Rating Scale (ALSFRS-R) score following both the first and second NCS transplantation. Monthly testing for the presence of human leukocyte antigen (HLA) antibodies matched to donor stem cells was consistently negative [31]. Donor DNA persisted for at least 921 days

Table 13.1 Review of registered clinical trials using stem cells for amyotrophic lateral sclerosis

Delivery route	Identifier	Location (sponsor)	Year (status)	Enrollment	Cell line	Dose
Autologous mesenchymal stem cells (MSC)						
Intraparenchymal	12947–29.3; 16454-pre21-823	Italy (Maggiore della Carità Hospital)	2001–2009 (Two, Phase I complete)	19	Bone marrow-derived MSC	Two to five injections, 6E5 cells/ul
Intrathecal	NCT02193893	Poland (Pomeranian Medical University Szczecin)	2010—(Phase I enrolling; invitation only)	50	Bone marrow-derived MSC	Not described
	NCT01363401	Korea (Corestem, Inc.)	2011–201 (Phase I/II complete)	71	Bone marrow-derived MSC	Two infusions, 26 day interval
	NCT01609283	USA (Mayo Clinic)	2012—(Phase I recruiting)	1	Adipose-derived MSC	Up to two infusions in separate procedures, 1E8 cells/infusion
	NCT02116634	Iran (Alzahra Hospital)	2014—(Phase I/II not open for recruitment)	5		One infusion 1E8 cells + 10 ml saline
Intravenous	NCT01759797	Iran (Royan Institute)	2012–2013 (Phase I complete)	6	Bone marrow-derived MSC	Not described
Intraventricular	NCT01771640	Iran (Royan Institute)	2013—(Phase I recruiting)	10	Bone marrow-derived MSC	Not described
	NCT01759784	Iran (Royan Institute)	2014—(Phase I not open for recruitment)	10	Bone marrow-derived MSC	Not described
Intrathecal and Intramuscular	NCT02017912	USA (Multicenter; Brainstorm-Cell Therapeutics)	2014—(Phase II recruiting)	48	Bone marrow-derived MSC, induced to secrete NTF	Not described
	NCT01777646	Israel (Hadassah Medical Organization; Brainstorm-Cell Therapeutics)	2012—(Phase II ongoing; not recruiting)	14	Bone marrow-derived MSC, induced to secrete NTF	Dose escalation; Group 1: 94E6 cells; Group 2: 141E6 cells; and Group 3: 188E6 cells

Intramuscular or intrathecal	NCT01051882	Israel (Hadassah Medical Organization; Brainstorm-Cell Therapeutics)	2011–2013 (Phase I/II complete)	24	Bone marrow-derived MSC, induced to secrete NTF	12 early stage patients, IM only: 24 sites with total of 24E6 cells; 12 progressive stage patients, IT only: total of 60E6 cells
Allogenic mesenchymal stem cells						
Intrathecal	NCT01494480	China (General Hospital of Chinese Armed Police Forces)	2012—(Phase II enrolling; invitation only)	30	Umbilical cord-derived MSC	4× administration via lumbar puncture, 3–5 days between treatments, two total treatments
	NCT01758510	Korea (Hanyang University Seoul Hospital)	2012—(Phase I recruiting)	18	Bone marrow-derived, HLA-haplo matched MSC	Three cohorts: 0.25E6 cells/kg, 0.5E6 cells/kg, 1E6 cells/kg. Two infusions, 28 day interval
Human fetal-derived neural stem cells						
Intraparenchymal	NCT01348451	USA (Multicenter; Neuralstem Inc.)	2009—(Phase I ongoing; not recruiting)	15	Spinal cord-derived	Up to 10 thoracolumbar and five cervical injections; 10 µl/injection, 1E4 cells/µl
	NCT01730716	USA (Multicenter; Neuralstem Inc.)	2013—(Phase II ongoing; not recruiting)	18	Spinal cord-derived	Up to 20 thoracolumbar and 20 cervical injections; Group A: 2E6 cells, Group B: 4E6 cells, Group C: 6E6 cells, Group D: 8E6 cells, and Group E: 8E6 cells + 8E6 cells
	NCT01640067	Italy (Azienda Ospedaliera Santa Maria)	2011—(Phase I recruiting)	18		Not described

(continued)

Table 13.1 (continued)

Delivery route	Identifier	Location (sponsor)	Year (status)	Enrollment	Cell line	Dose
Autologous hematopoietic stem cells						
Intraparenchymal	NCT00855400	Spain (Fundacion para la Formacion e Investigacion Sanitarias de la Region de Murcia)	2007–2010 (Phase I complete)	11	Bone marrow-derived mononuclear cells	Not described
Intraparenchymal or intrathecal	NCT01254539	Spain (Fundacion para la Formacion e Investigacion Sanitarias de la Region de Murcia)	2010—(Phase I/II ongoing; not recruiting)	63	Bone marrow-derived mononuclear cells	Phase I: T3–T4 intraparenchymal injections only; Phase II: intrathecal only
Intrathecal	NCT01933321	Mexico (Hospital Universitario; Dr. Jose E. Gonzalez)	2012—(Phase II/III ongoing; not recruiting)	14		Not described
Intrathecal and intramuscular	NCT01984814	India (Neurogen Brain and Spine Institute)	2008–2013 (Phase II complete)	57	Bone marrow-derived mononuclear cells	Not described
Not specified	NCT02242071	India (Neurogen Brain and Spine Institute)	2008—(Phase I recruiting)	200	Bone marrow-derived mononuclear cells	Not described

Table 13.2 Review of published clinical trials, pilot studies, and observational studies using stem cells for amyotrophic lateral sclerosis

Year	Location	Enrollment	Delivery route (cannula)	Dose (number of patients)	Follow-up	Outcome measures	Observed adverse events (number of patients)	Reference
Autologous mesenchymal stem cells (MSC)								
2014	South Korea	37	Intrathecal (L2–L3 lumbar puncture)	Two 1E6 cells/kg injections, 1 month interval	6 months	Identify biological markers to predict response to therapy; changes in ALSFRS-R	Mild fever (11); general myalgia (9); low back pain (4); headache (4)	[26]
2012 2008	Italy	9 and 10	Intraparenchymal (table-stabilized 18G Hamilton syringe targeting thoracic central cord region)	Two to five injections; median of 75E6 cells (range: 11E6–120E6 cells), 25 µl/injection	9 years	Safety; changes in ALSFRS and FVC	Intercostal pain irradiation (4); transient pain (7); leg sensory dysesthesia (6); light-touch impairment in one leg (4) or sacral region (1); tingling in one leg (6)	[27]
2012	South Korea	1	Intraventricular (Ommaya reservior)	1E6 cells/kg suspended in autologous CSF	Not described	Case report	None reported	[28]
2010	Spain	9	Intrathecal and intravenous (2) or intrathecal only (7)	Not described	24 months	Observational study; ALSFRS-R and FVC analyzed	Intense back pain (1) managed with morphine derivatives for 72 h after lumbar puncture	[29]
2010	Israel	19	Intrathecal and intravenous (9) or intrathecal only (10)	IT: mean 54.7E6 (SD 17.4E6) cells in 2 ml normal saline; IV: mean 23.4E6 (SD 6E6) cells in 2 ml normal saline	6–25 months	Safety; changes in ALSFRS; immunological analysis; MRI pathology	Fever (11); headaches (5); leg pain (2); dyspnea (1)	[30]

(continued)

Table 13.2 (continued)

Year	Location	Enrollment	Delivery route (cannula)	Dose (number of patients)	Follow-up	Outcome measures	Observed adverse events (number of patients)	Reference
Human fetal-derived neural stem cells								
2014	USA	15	Intraparenchymal (patient-stabilized 29G injection platform and microinjector pump)	Up to 10 thoracolumbar and five cervical injections; 10 µl/injection, 1E4 cells/µl	24 months	Safety; changes in ALSFRS-R, Neuropathic Pain Scale, Ashworthy Spasticity Scale, hand strength, respiratory parameters	Transient radicular-type pain and/or sensory abnormalities (several); dural fistula and repaired CSF leak (1); wound dehiscence (1)	[31]
Human fetal-derived olfactory ensheathing cells								
2010	Spain	3	Intracerebral, corona radiata	100 µl volume containing 2E6 cells	24 months	Observational study; ALSFRS-R and FVC analyzed	Fever and deterioration in consciousness (1) presented 48 h after surgery, lasting 5 days	[29]
2008	China	35	Intracerebral, corona radiata	Treatment group: 2E6 cells bilaterally (15)	4 months	Functional decline measured by ALSFRS	None reported	[32]
Autologous hematopoietic stem cells								
2012	Spain	11	Intraparenchymal (T2–T4 central cord region via 22G lumbar puncture needle)	Median of 462E6 BMNC (range 138.00–602.87) resuspended in 2 ml normal saline; two total injections, each 1 ml BMNC-saline	12 months	Safety; histopathological signs of cellular neurotrophism (GDNF)	Constipation (10); surgical wound pain (7); temporary intercostal pain (5); transient intracranial hypotension (3); hypoesthesia (7); paresthesia (4); dysesthesia (2)	[33]

2012	Mexico	67	Intracerebral (frontal motor cortices via stereotaxy or neuronavigation and Hamilton syringe)	2.5E5—7.5E5 cells suspended in 0.3 ml autologous CSF (10) or 3.0E6–5.0E6 cells suspended in 0.3 ml autologous CSF (57)	24 months	Safety; changes in ALSFRS-R	Myocardial infarction with subdural hematoma resulting in death (1); transient skin pain (several); minor scalp pain (several); headache (several)	[34]
2010	Israel	35	Subcutaneous Neupogen (G-CSF) stimulated	Treatment group: 5 ug/kg/day Neupogen for 4 days (one cycle), repeated every 3 months for 4 cycles (17); Control group: normal saline placebo (18)	12 months	ALSFRS-R; VC, manual muscle strength, compound muscle action potential amplitudes, neurophysiological index, McGill QoL	Bone and muscle pain after injection (2)	[35]
2009	Turkey	13	Intraparenchymal (C1–C2), intrathecal, and intravenous	Spinal cord: 0.1 ml/injection; brainstem: 3 ml (10E6 cells); subarachnoid space: 1.5 ml (5E6 cells); intravenous: 1.5 ml (5E6 cells)	12 months	Safety; changes in bulbar and Norris scores; electroneuro myography; absence of MRI-indicated pathology	None reported	[36]
2008	Canada	8	Subcutaneous Neupogen (G-CSF) stimulated, intravenous reinfusion	300–600 ug/day Neupogen for 5–6 days; 3.3E6 (SD 2.0E6) $CD34^+$ cells/kg reinfused on day 7	6 months	Safety; changes in ALSFRS-R, FVC, manual muscle testing; absence of MRI-indicated pathology	Deep venous thrombosis (1)	[37]

(continued)

Table 13.2 (continued)

Year	Location	Enrollment	Delivery route (cannula)	Dose (number of patients)	Follow-up	Outcome measures	Observed adverse events (number of patients)	Reference
2001	USA	3	Subcutaneous Neupogen (G-CSF) stimulated, intrathecal reinfusion	1E8 cells over 2 days (1); 2E7 cells reinfused at L3–L4 and into cisterna magna (33 % total cells) (1); 1E8 cells reinfused to C1–C2 intrathecal space (40 % total cells) and lumbar intrathecal space (60 % total cells) (1)	6–12 months	Case report	Transient 2 h loss of sensation in lower limbs (1)	[38]
Allogenic hematopoietic stem cells								
2008	USA	6	Intravenous infusion of G-CSF stimulated HSCs	Not described	Not described	Percent engraftment, toxicity, graft rejection, disease progression, and survival using AALS scoring	Cutaneous acute graft versus host disease (GVHD) (2); limited chronic GVHD (3)	[39]

post-transplant, and definitive evidence for donor cell survival was found in a female patient (Fig. 13.2). A Phase II study with classic dose escalation delivery to the cervical spinal cord is ongoing (ClinicalTrials.gov ID: NCT01730716). We believe the risk escalation model represents a novel design for Phase I safety trials that involve multiple parameters likely to affect morbidity.

Another group is investigating the use of NSCs as a therapeutic approach for ALS patients in the clinic (ClinicalTrials.gov ID: NCT01640067). Similar to our study, this study at Azienda Ospedaliera Santa Maria in Terni, Italy, will be utilizing a risk escalation model for patient recruitment. This Phase I study will deliver fetally derived NSCs to 18 patients by direct injection into the spinal cord.

The Future of Neural Stem Cells

Past and ongoing trials of NSCs have used unmodified NSCs. Although they have shown some efficacy in animal models, there is clearly room for improvement. Klein et al. transduced human fetal cortical NPCs with a lentivirus expressing GDNF. Four unilateral injections of 180,000 cells were injected into the lumbar spinal cord in presymptomatic rats. At 6 weeks post-transplantation, cells were found throughout the lumbar cord on the injected side and motor neuron loss was delayed. However, motor function was not improved, likely due to a failure to preserve neuromuscular junctions. The parental cell line failed to provide any benefit [41]. Park et al. modified human fetal forebrain NSCs to express BDNF, IGF-1, VEGF, neurotrophin-3, or GDNF and injected them into the cisterna magna. Cell migration into the spinal cord was low and little efficacy was observed [42]. In contrast, injection of VEGF-secreting HB1.F3 cells (an NSC line produced from human fetal telencephalon by transduction with a retroviral vector encoding v-myc) intrathecally into presymptomatic SOD1 mice delayed disease onset, improved motor function, and increased life span better than the parental cell line [43].

Although Martin et al. found that NSCs from mice with ALS were inferior to cells from healthy mice, [10] autologous NSCs may still be a useful route to therapy. Nizzardo et al. investigated the therapeutic potential of induced pluripotent stem (iPS) cells derived from healthy human fibroblasts and differentiated into NSCs that were ALDH high, VLA4+, and low orthogonal light scattering. Beginning at 90 days of age, SOD1 mice received weekly intravenous (IV) injections of 1 million cells or three weekly intrathecal injections of 1 million cells each. Life span was increased, with the IV group living 23 days longer and the intrathecal group living 10 days longer [44]. Although they started with fibroblasts from healthy individuals, one could imagine producing iPS cells from ALS patient fibroblasts and correcting the mutation before cell infusion.

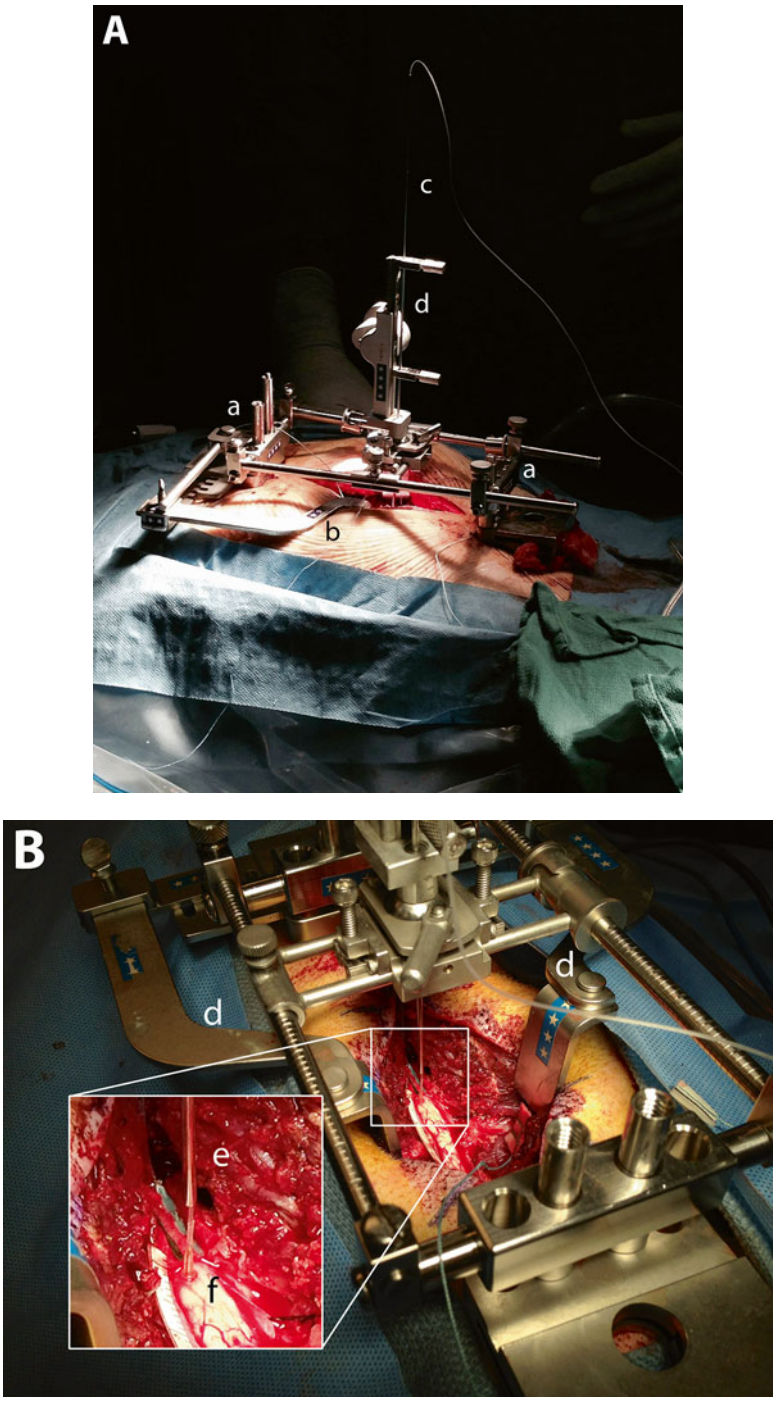

Fig. 13.1 Patient-stabilized microinjector platform with immobilized floating cannula system. This system was developed for safe and accurate spinal cord microinjections of cells or gene therapy vectors. With the patient lying prone, the spinal cord rises and falls with each breath, presenting a risk of spinal cord damage for table-mounted microinjection apparati. In contrast, a patient-stabilized platform offers improved stability by remaining immobilized relative to the

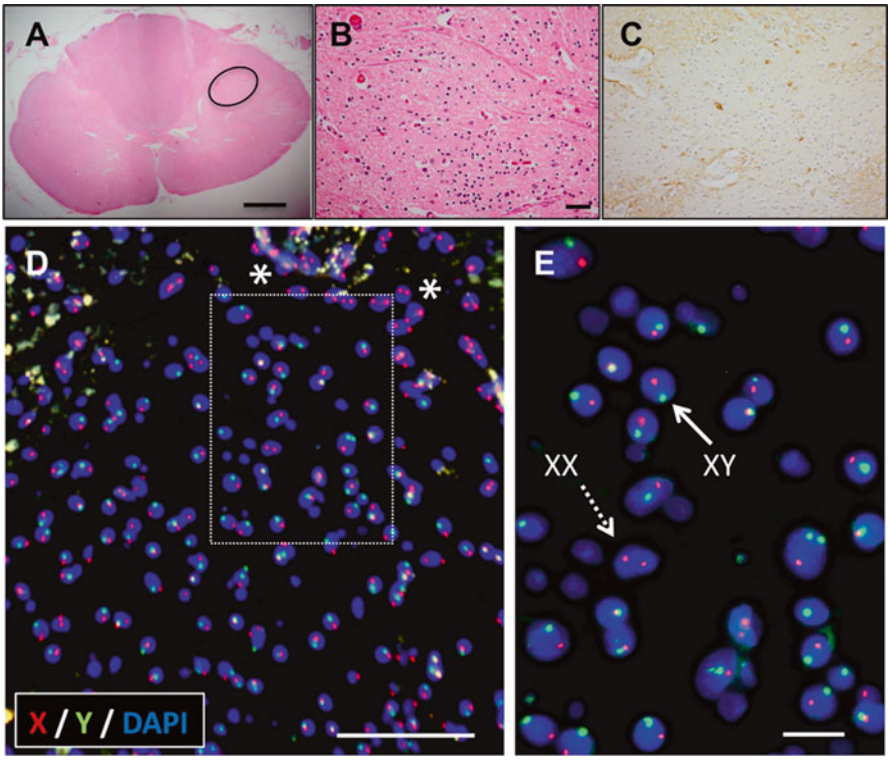

Fig. 13.2 Survival of grafted cells was evaluated in a female ALS patient that received unilateral injections of NSCs into the cervical spinal cord. (**a**) A spinal cord section stained with hemotoxilin is shown. (**b**) Higher magnification of the region circled in A shows a "nest" of putative NSCs. (**c**) These cells are GFAP negative (brown stain). Taking advantage of the fact that the donor cells are male and the patient is female, fluorescence in situ hybridization was performed to detect the X (*red*) and Y (*green*) chromosomes to discriminate between donor (X/Y) and recipient (X/X) cells (D—low magnification; E—higher magnification of the area outlined in D). Many donor cells were found in the "nest" of NSCs, which is surrounded by recipient cells (*asterisks*).

Fig. 13.1 (continued) spinal cord during cardiovascular pulsation, ventilation-associated cord movement, or inadvertent patient movement [77]. (**a**) The platform is shown mounted on an ALS patient for NSC injection. Posts (a) provide bony spinal attachment to the lamina or occiput. Integrated, self-retaining retractors (b) maintain access to the spine. The floating cannula (c) is fed through the injection stage (d) and consists of a flanged 30-gage beveled needle connected to flexible tubing. (**b**) The platform is shown mounted for thoracolumbar injection into the porcine spinal cord. Inset, the flexible tubing is ensheathed with a rigid outer cannula (e) that sits against the needle flange during cord penetration and is retracted distally from the needle during injection (f). This retraction allows the flexible tubing to bend and move with spinal cord displacement, reducing sheering and the likelihood of neurological morbidity

Mesenchymal Stem Cells

Evidence from Animal Studies

While NSCs have generally been delivered directly into the spinal cord, MSCs have been delivered in a number of ways, given their ability to home to sites of tissue damage. IV injection is likely the safest method, although it will require higher doses to overcome the loss of cells engrafting to non-CNS tissues. Uccelli et al. delivered 10^6 bone marrow-derived MSCs to presymptomatic SOD1 mice. Life span was extended by 17 days, motor function improved, and astrogliosis and biochemical markers of disease were attenuated [45]. Cell survival was low 35 days post-transplant, pointing to the possible need for re-dosing. Sun et al. gave injections of 10^6 MSCs derived from amniotic sac membrane at 12, 14, and 16 weeks of age to presymptomatic SOD1 mice by jugular vein injection with similar results [46]. Kim et al. administered 10^6 adipose-derived MSCs to presymptomatic female SOD1 mice. Life span increased by 9 days [47]. In contrast, when given at symptom onset, 2×10^6 adipose-derived MSCs slowed motor function loss but failed to increase life span [48].

Delivery into the cerebrospinal fluid represents a compromise between less targeted but safer IV delivery and more targeted but more risky parenchymal injection. Using intrathecal delivery, Boido et al. gave SOD1 mice 300,000 human bone marrow MSCs [49], while Boucherie et al. delivered 2×10^6 rat bone marrow MSCs [50]. Both studies found that this therapy slowed the development of muscle paralysis and preserved motor neurons. The latter also showed an improvement in life span of 16 days.

Morita et al. delivered 400,000 rat bone marrow MSCs into the fourth ventricle of SOD1-Leu126delTT mice 6 weeks before disease onset. Interestingly, the only benefit was an increase in disease duration in female mice [51]. The reason for this difference between the genders was unclear, but sex differences have been described previously in SOD1 models [52]. Kim et al. injected 200,000 adipose-derived MSCs into the lateral ventricle of presymptomatic SOD1 female mice. Life span was increased by 24 days, and 0.7 % of the grafted cells were found in the spinal cord gray matter 4 weeks post-transplantation [47].

Three studies have investigated injection into the cisterna magna. Habisch et al. evaluated human bone marrow MSCs, as well as MSCs differentiated into NSCs. 100,000 cells were injected into presymptomatic SOD1 mice. Neither cell type extended life span, but engraftment in the spinal cord 10 days post-transplant was low [53]. Kim et al. reported two studies investigating bone marrow MSCs derived from ALS patients. In the first study, they delivered up to 10^6 cells into presymptomatic SOD1 mice. With the highest dose, life span increased by 6 days but symptom onset was not delayed. Efficacy dropped with decreasing dose [54]. In their second study, patients in their clinical trial were partitioned by their revised ALS functional rating scale (ALSFRS-R) score. Those that showed a rate of decline slower than it was before transplant were labeled “responders.”

Cells from one patient from each group were tested in SOD1 mice (10^6 cells/kg via cisterna magna injection). Although there were significant differences between mice receiving cells from a responder and PBS controls, there were no significant differences between animals receiving responder or nonresponder MSCs [26]. Unfortunately, the study did not include MSCs from healthy donors, which would help determine if MSCs from ALS patients are less potent than those from health donors.

Like NSCs, MSCs have also been evaluated for parenchymal delivery. In presymptomatic SOD1 mice, 10^5 human bone marrow MSCs were delivered by injection into the lumbar spinal cord. Motor neuron survival improved and gliosis was reduced [55]. Like Morita et al., females responded more favorably to the therapy. In contrast to the SOD1 data, mdf mice receiving 5×10^5 bone marrow MSCs showed no improvement [56].

Forostyak et al. investigated a combination of two delivery methods of rat bone marrow MSCs in early symptomatic SOD1 rats: two injections of 5×10^4 into the spinal cord at T10 and 2×10^6 cells delivered IV. Some improvement in motor function was observed, along with increased motor neurons survival. Survival was increased by 11 days [57]. Unfortunately, since the authors did not evaluate the two delivery methods separately, it is unclear whether the combined therapy is superior to the individual delivery methods.

Differentiation and Survival

In vitro, MSCs exposed to particular cocktails of cofactors have been shown to transdifferentiate into neural lineages. This finding suggested that MSCs might not only deliver trophic factors to the diseased spinal cord, but also integrate into the existing neural structure. Zhao et al. further found that MSCs cultured in extracts from the spinal cords of SOD1 mice took on a neuronal appearance and expressed several neuronal markers [58]. However, most studies have found little evidence of transdifferentiation in vivo, with less than 1 % of cells expressing neuronal, astrocyte, or oligodendrocyte markers [46, 48, 55, 57]. In one isolated study, up to 31 % of donor cells near motor neurons expressed GFAP, an astrocyte marker [50].

Questions also remain regarding the persistence of MSCs. Uccelli et al. found that cell survival was low at 35 days post-transplant [45]. BrainStorm Cellular Therapeutics has tested autologous MSCs they call “NurOwn” in Phase I/II and Phase IIa clinical trials. These cells are induced to secrete higher levels of GDNF, BDNF, VEGF, and hepatocyte growth factor by manipulating the culture conditions prior to transplantation. However, they have found that the cells survive only a few weeks in vivo [59]. Unfortunately, since the improvements in survival for most animal models of ALS have been measured in terms of days or weeks, it is unclear from most studies what the persistence of MSCs is in the context of this disorder.

Clinical Trials of MSCs

With the immunomodulating properties of mesenchymal stem cells shown in preclinical studies, Karussis et al. evaluated the safety and immunological effects of intrathecal and IV delivery of autologous MSCs in 19 progressive ALS patients. A mean of 54.7×10^6 cells was administered intrathecally to all 19 patients. Nine of these patients also received IV delivery (mean 23.4×10^6 cells) [30].

Patient ALS Functional Rating Scale (ALSFRS) scores deteriorated slightly during the 2-month observation period before MSC intervention. During the first 6 months of postoperative observation, the mean ALSFRS score remained stable. Due to the short preoperative observational period and the variability in disease progression, no significant improvement in disease trajectory was observed. Immunological analysis was performed on five patients who underwent both intrathecal and IV MSC delivery. Most notably, 24 h after MSC administration they observed a 72 % increase in the proportion of $CD4^+$, $CD25^+$ regulatory T cells and a 30–60 % reduction in the number of $CD86^+$, $CD83^+$, and HLA-DR^+ myeloid dendritic cells. Activated $CD40^+$ antigen-presenting cells and activated lymphocytes were also reduced. These changes were observed as early as 4 h after transplantation, but the longevity of these effects was not studied [30]. Though controlled study with longer follow-up is needed, this clinical trial demonstrates the possibility of using MSCs for systemic immunomodulation.

In 2008, an Italian group published results from a pilot study of nine patients who underwent thoracic (T7–T9) intraparenchymal injection of autologous bone marrow-derived MSCs via a table-stabilized Hamilton syringe (median dose 32×10^6 cells, range 7×10^6–152×10^6 cells, mean 57×10^6 cells delivered). Due to safety concerns, only patients with severe lower limb disability were enrolled. Baseline data for ALSFRS and forced vital capacity (FVC) were collected for 6 months before intervention. Patients were monitored for at least 4 years after intervention and no major adverse events were observed. The authors concluded the procedure was safe because disease progression did not accelerate relative to baseline. A slowing of the linear decline of FVC and ALSFRS was observed in five of these patients after intervention, though disease progression was generally variable [60].

This small pilot study encouraged further investigation, and a subsequent Phase I clinical trial enrolled 10 patients (ClinicalTrials.gov ID: 16454-pre21-823). These patients had less advanced disease relative to the pilot study [median ALSFRS score: 33 (range: 26–39); median FVC: 99 % (range: 51–117 %)]. After intervention, patients were assessed every 3 months for up to 24 months. No serious adverse events were observed. No significant modifications to ALSFRS or FVC measures or decline were observed between the preoperative and postoperative periods. MR-imaging comparisons in all patients revealed a slight, hyperintense segmental increase of spinal cord volume at cell injection sites which resolved over time. Long-term monitoring of the 19 patients from these two studies found no evidence of tumor formation or abnormal cell growth at injection sites [27].

It was proposed that inclusion criteria and site of transplantation could explain differences in postoperative ALSFRS and FVC measures between the first and

second study. Clinical and neurophysiological long-term assessment showed a predominant lower motor neuron involvement in 3 of 5 patients whose prognosis improved or stabilized in the initial pilot study [61]. If this hypothesis is true, it would highlight the need to adequately coordinate disease location, injection sites, and clinical measures.

Kim et al. sought to identify patient-specific biological markers that may correlate to clinical efficacy in an open-label, single-arm clinical study. After a 3-month baseline lead-in period, 37 patients received lumbar intrathecal delivery of autologous bone marrow-derived MSCs (10^6 cells/kg) in two doses separated by 1 month. Patients were followed for 6 months after intervention. Revised ALSFRS (ALSFRS-R) scores were evaluated every 3 months and compared to baseline disease progression rate. No serious adverse events occurred, and all nonserious adverse events were transient (Table 13.2).

The authors grouped patients based on disease trajectory after therapy: nonresponders, who progressed at the same or faster rate compared to baseline; and responders, who had ALSFRS-R scores higher than baseline or progressed at a slower rate [26]. Although the mean decrement in the ALSFRS-R score was smaller in responders versus nonresponders, there was no significant difference at 6 months. Nine trophic factors were quantified in MSC cultures from both groups. VEGF, angiogenin, and transforming growth factor-β were somewhat elevated (10–20 %) in MSC cultures of responders compared to nonresponders [26].

The authors hypothesized that the trophic support of responder MSCs better enabled motor neuron survival compared to nonresponder MSC trophic support. As we described above, the authors sought to confirm the effectiveness of responder MSCs by transplanting 10^6 MSCs from one responder and one nonresponder into the cisterna magna of SOD1-G93A transgenic mice at day 60. No significant difference in disease progression was noted between the two groups [26]. Although they failed to show differences between the two groups, the idea of prescreening stem cells for trophic factor secretion prior to treatment is an interesting one and should be the focus of future studies.

This group also provided a single patient case report of cerebrospinal fluid (CSF)-delivery via intraventricular injection using an Ommaya reservoir [28]. Though further investigation is needed, this method could allow for repetitive and reliable CSF-targeted injections.

To date, no clinical data have been published on the use of allogeneic mesenchymal stem cells in patients with ALS. However, two clinical trials are ongoing (Table 13.1).

The Future of MSC Therapy

Like NSCs, the future of MSC-based therapies will likely revolve around increasing their secretion of neurotrophic factors and improving their homing potential to the spinal cord. Suzuki et al. transduced bone marrow MSCs with a

lentivirus expressing GDNF and injected them into muscles damaged by treatment with bupivacaine hydrochloride. The GDNF-expressing MSCs led to increased muscle innervation relative to untreated rats and extended life span. However, there was no statistical difference between modified and unmodified MSCs [62]. Suzuki's group built upon this study, testing four different neurotrophic factors: GDNF, VEGF, IGF-1, and BDNF. Expression was greatest with the GDNF and VEGF constructs. Combined overexpression of GDNF and VEGF in MSCs led to longer survival compared to unmodified MSCs or to either factor alone, demonstrating enhanced potential [63].

Knippenberg et al. modified MSCs to overexpress glucogon-like peptide 1(GLP1), a molecule that protects against excitotoxicity and has antioxidant capacity. The authors encapsulated the cells and injected them into the ventricles of presymptomatic SOD1 mice. These cells delayed disease onset by 15 days and increased survival by 13 days. Unmodified cells were not investigated, making it unclear what role the transgene played. In addition, the capsules could not be recovered, and cell survival could not be ascertained [64].

Choi et al. transduced MSCs with a retrovirus expressing neurogenin 1 (Ngn1) to reprogram the cells to a neural fate. These cells expressed neural marker in vitro but failed to do so in vivo. These cells showed greater migratory ability than unmodified MSCs. IV delivery of 10^6 cells to symptomatic SOD1 mice increased life span by 7 days and significantly slowed motor function loss relative to untreated and MSC-treated controls. Interestingly, intervention 6 weeks before symptom onset only led to a 3-day increase in life span. The authors hypothesized that the cells migrated more efficiently to the spinal cord when the disease was more advanced, although they did not quantify spinal cord engraftment in both cohorts [65]. A competing hypothesis would be that these MSCs do not persist long term, and thus early delivery may fail to target the disease at the correct time.

Bone Marrow Stem Cells

Evidence from Animal Studies

Like MSCs, bone marrow-derived stem cells can both permit the use of autologous cells and avoid the ethical concerns of using fetal-derived cells. A small number of animal studies have investigated this treatment paradigm. Corti et al. gave 3×10^7 mouse bone marrow cells (BMCs) to 4-week-old (presymptomatic) SOD1 mice following 800 rad of radiation. Life span was improved by ~13 days. Motor neuron counts and axon number were higher, and motor function loss was delayed by 14 days. Some "donor-derived neurons" were found, but DNA analysis suggested that they were the result of cell fusion events rather than transdifferentiation. 27 % of microglia were donor-derived. Donor-derived cells were also found in skeletal and heart muscle, expressing myocyte markers [11].

In a second study, Corti et al. evaluated the efficacy of c-kit$^+$ (CD117$^+$) BMCs. These cells do not express hematopoietic, mesenchymal, or endothelial markers, but do express Oct-4 at high levels and the astrocyte glial glutamate transporter protein (GLT1). 70-day-old SOD1 mice received 10^6 cells by tail vein injection. Cells were found in both the gray matter and the white matter at end stage. 71 % of the cells migrated to the ventral half of the cord. Donor cells did not express neural or glial markers, including GFAP, but they maintained expression of GLT1. Survival was increased by 17 days, symptom onset was delayed, and disease progression was slower. The therapy elevated GLT1 levels, and the authors suggest that this could have an effect similar to the transplantation of astrocytes [66]. Knockdown of GLT1 reduced the effectiveness of the cells [14].

Ohnishi et al. examined bone marrow transplantation (BMT) in the context of symptomatic SOD1 mice. Early symptomatic SOD1 mice received two doses of 5.5 Gy or 6.0 Gy of gamma radiation prior to BMT with 6×10^7 cells. Engraftment levels for both types of BMCs were greater than 90 % in the bone marrow. Life span in animals receiving wild-type BMCs was increased by 14 days, and the rate of motor function was slowed. There were significant number of donor cells in the ventral cord. Some expressed Iba1, a microglial marker, but none expressed neuronal, astrocytes, or oligodendrocyte markers [12].

Two studies have evaluated the efficacy of injected BMCs directly into the spinal cord of muscle deficient (mdf) mice. Pastor et al. injected 1 million mouse BMCs into the ventral horns of symptomatic mice. No significant improvement was found [56]. In contrast, Cabanes et al. purified CD117$^+$ bone marrow cells to enrich for hematopoietic stem cells (HSCs). 300,000 cells were injected into the lumbar cord. Cells survived for at least 10 weeks and spread up to 800 μm from the injection site. Grafted cell numbers increased over time, suggesting that they could multiply within the cord. 5–10 % of cells in the grafted area were donor-derived microglia. HSCs did not appear to differentiate into neurons, astrocytes, or oligodendrocytes. Improvement was observed in motor function, but survival was not evaluated [67]. These studies highlight the fact that whole bone marrow is a heterogeneous mixture of cells. Purification of multipotent stem cells is likely needed in clinical trials to account for possible differences in the size of the stem cell population and to ensure equivalent dosing between individuals.

Clinical Trials of Bone Marrow Stem Cells

Martinez et al. investigated intracerebral delivery of autologous HSCs in the largest uncontrolled, open-label clinical trial for this cell line yet undertaken. Sixty-seven patients with either bulbar-onset ($n = 19$), spinal-onset ($n = 47$), or bulbospinal-onset ($n = 1$) were enrolled. 300 ug of Neupogen (human granulocyte colony-stimulating factor, G-CSF) was administered subcutaneously for 3 days to obtain peripheral blood mononuclear cells. Isolated autologous CD133$^+$ cells were resuspended in autologous CSF and implanted bilaterally into the frontal motor cortices (Table 13.2).

The delivered dose ranged from 2.5×10^5–7.5×10^5 ($n = 10$) to 3.0×10^6–5.0×10^6 total cells ($n = 57$). Adverse events were monitored for 1 month after intervention. One death, due to myocardial infarction with subdural hematoma, was considered to be associated with the procedure [34].

Martinez et al. suggested that preliminary results depict a trend toward disease stabilization. Survival at 1 year was 90 and 52 % at 2 years after intervention. Mean long-term survival rate was 40 months from diagnosis, determined from at least a 2-year follow-up period. At present, this group is investigating intrathecal delivery of autologous HSCs in 14 patients (ClinicalTrials.gov ID: NCT01933321).

Previously, Nefussy et al. suggested that cells mobilized into the peripheral blood by G-CSF could slow down disease progression, without additional intervention or CNS transplantation. G-CSF has also been shown to be neuroprotective by a direct antiapoptotic effect [68]. In a double-blind, controlled clinical study, 17 patients were given Neupogen (5 ug/kg/day) subcutaneously for four consecutive days. This cycle of 4 days was repeated every 3 months for a total of four cycles, with no significant adverse events observed. Eighteen patients received placebo injections. The day after treatment, patients who received G-CSF had 3.8 times more white blood cells and 12.3 times more $CD34^+$ cells compared to patients who received placebo. However, no significant improvement in disease progression was observed.

Similar results were produced in a pilot study of 8 patients receiving G-CSF stimulation, collection, and reinfusion of mobilized peripheral blood stem cells (PBSCs). Patients received Neupogen (weight-dependent dose, 300–600 μg) subcutanteously for 5–6 days. After leukapheresis, autologous PBSCs were reinfused at 3.3 ± 2.0 (range 1.5–$7.6) \times 10^6$ cells/kg IV. A 6-month follow-up period revealed no significant changes to ALSFRS-R, FVC, and manual muscle testing (MMT) compared to preoperative status [37]. It is not clear what additional benefit the authors expected to gain by removing the cells and then reinfusing them the following day.

Blanquer et al. reported preliminary findings from an open, single-arm Phase I trial assessing the safety of intraparenchymal infusion of autologous, BM-derived mononuclear cells in patients with spinal-onset alS (ClinicalTrials.gov ID: NCT00855400). Autologous mononuclear stem cells were collected from bone marrow harvested from the posterior iliac crests. After 6 months of preoperative observation, 11 patients underwent thoracic T3–T4 laminectomy and stem cell infusion through posterior spinal cord funiculus (Table 13.2). Patients were monitored quarterly for 1 year and assessed for rate of disease progression using ALSFRS, FVC, Medical Research Council scale, and the Norris scale. No serious adverse events were reported during follow-up, and no acceleration in the rate of decline was observed during the postoperative follow-up [33].

Interestingly, pathology and histological analysis of 4 patients revealed a significantly greater number of motor neurons in treated spinal cord segments compared to untreated segments (4.2 ± 0.8 motor neurons/section and 0.9 ± 0.3 motor neurons/section, respectively). In treated segments, $CD90^+$ cells surrounded motor neurons. Motor neurons had substantially fewer ubiquitin deposits, and they showed fewer signs of degeneration when compared to untreated segments [33].

The migration of donor cells toward motor neurons was also demonstrated in preclinical animal studies [67], and a GDNF-mediated neurotrophic effect was hypothesized to favor survival of host motor neurons. The results suggest that bone marrow-derived mononuclear cells can provide neurotrophic factor delivery in humans, warranting further investigation in the clinic. A Phase I/II trial by this group is ongoing (ClinicalTrials.gov ID: NCT01254539). This study will investigate the effect of intraparenchymal and intrathecal injection of autologous mononuclear cells on FVC measurements in 63 patients.

A diverse array of transplantation strategies has been implemented to deliver hematopoietic stem cells to the central nervous system. However, little is known about the possible benefits of combining different routes of therapy to potentially treat different aspects of the disease. In a Phase II study, one group examined the safety and efficacy of delivering HSCs by combining several different delivery strategies targeting the brain and spinal cord. Thirteen patients with bulbar involvement and severe loss of movement were enrolled. Autologous mononuclear cells were collected after bone marrow aspiration from the iliac crest. Patients underwent cervical laminectomy at C1–C2 levels and intraparenchymal injection toward the anterior cord region (dose not described). Ten million cells were then injected into a Gel Foam stem cell storage material and used to cover the exposed cord, lower cranial nerve, and brain stem. Additionally, upon dural closure, 5 million cells were injected into the intrathecal space at operation site and 5 million cells were administered IV. After 12 months of follow-up, 9 patients maintained better bulbar and Norris scores and 1 patient remained stable compared to preoperative scores. Pre- and postoperative electroneuromyography measurements confirmed reinnervation in 7 of these patients [36].

Only one study has investigated allogeneic hematopoietic stem cells. Six patients received total body irradiation followed by infusion of HSC obtained from HLA-identically matched sibling donors. The immunosuppressant regimen consisted of IV tacrolimus (0.03 mg/kg/day) tapered at post-infusion day 30 and IV methotrexate (5 mg/m^2) at days 1, 3, 6, and 11. Four patients reached 100 % engraftment. However, only one patient progressed at a slower overall rate compared to matched controls. Postmortem analysis revealed that peripheral cells from a donor can enter the CNS and engraft at sites of motor neuron pathology, and low levels of engraftment correlated with poor migration of cells to the CNS. Though no clinical benefit was observed, such cells may provide a cellular vehicle for noninvasive therapeutic approaches in future studies [39].

The Future of Bone Marrow Stem Cells

Similar to NSCs and MSCs, research is being done to find ways to enhance the therapeutic value of BMCs. Terashima et al. have investigated the use of culture conditions to change the transcriptional profile of BMCs. They exposed mouse BMCs to either stem cell factor (SCF), a ligand for c-kit, or FMS-like

tyrosine kinase 3 (flt3), a hematopoietic stem cell differentiation factor. 8-week-old (presymptomatic) female SOD1 mice received 9 Gy of radiation followed by tail vein injection of 4×10^6 untreated or treated BMCs. Untreated BMC extended life span by about 2 weeks, but flt3-treated BMCs had no effect. SCF-BMC-treated mice exhibited the greatest improvement, extending median life span by about 4 weeks and improving motor function. SCF-BMCs also had higher levels of engraftment in the spinal cord, where they adopted a microglial fate. Inflammatory markers TNF-alpha and IL-1beta exhibited the greatest reduction in the SCF-treatment group. Interestingly, the SCF-treated BMCs expressed GLT-1, suggesting that they may be acting, in part, to reduce glutamate-induced neurotoxicity [13].

Olfactory Ensheathing Cells

Evidence from Animal Studies

Three animal studies have investigated the efficacy of OECs for the treatment of ALS. Morita et al. reported on mouse OECs tested in 13-week-old (presymptomatic) SOD1 mice. Cells (400,000) were injected into the fourth ventricle. Although the cells survived at least 100 days, no efficacy was observed [51].

In contrast, Li et al. demonstrated efficacy when OECs were delivered to the white matter. In the first study they injected 10^5 rat OECs into the dorsal funiculus of the thoracic spinal cord of presymptomatic SOD1 rats. Four weeks after transplantation cells were found in both the gray and white matter, up to several millimeters from the injection site. Cell migration was most prominent along neuronal fibers, and grafted cells appeared to be able to remyelinate axons. Life span was increased by 7 days. There was a small improvement in motor function during the symptomatic period, and motor neuron loss was delayed [69]. In the second study, they injected 5×10^5 rat OECs into the corona radiata of presymptomatic SOD1 rats. Life span increased 15 days with therapy and motor deficits were delayed [70].

Clinical Trials of Olfactory Ensheathing Cells

Fifteen patients underwent fetal-derived OEC transplantation in a controlled, but unblinded clinical study. The primary endpoint of this trial was to study changes in the rate of ALSFRS decline in the treatment group compared to 20 control subjects. Treatment group patients received 2×10^6 cells injected bilaterally into the corona radiata involving the pyramidal tracts of the frontal lobes. No immunosuppressant was given. A trained home caregiver generated ALSFRS data over a 4-month post-operative follow-up period [32].

During the follow-up period, the ALSFRS total score decreased by 0.07 ± 4.18 points in the treatment group and 6.12 ± 5.49 points in the control group. No significant difference was found between the rates of ALSFRS deterioration for treated and control group subjects during the first 2 months of follow-up. However, functional deterioration was significantly slower for the treatment group during the last 2 months of the 4-month follow-up. The authors proposed that the delayed positive trend may be explained by axon remyelination of the corticospinal and/or corticobulbar tract [32].

Umbilical Cord Blood

Clinical trial results of UCBs have not yet been published for ALS. However, there have been five reports examining UCB therapies in animal models. In the earliest study, approximately 7-week-old presymptomatic SOD1 mice underwent NK cells depletion prior to receiving 400 or 800 cGy of gamma radiation and 33 or 70 million UCB-mononuclear cells. Treatment with 33 million UCBs increased life span from up to 23 days, while 70 million cells increased life span up to 38 days. Interestingly, animals that died earlier tended to have cells from a single donor, while longer-lived animals tended to have multiple donors, suggesting that not all sources are equally robust [71]. The fact that the authors needed to pool UCBs from multiple donors to treat a mouse seems to be a major hurdle for future clinical application of these cells. Garbuzova-Davis et al. evaluated lower doses of UCBs. They gave presymptomatic SOD1 mice 10^6 UCB cells by jugular vein infusion. Treatment delayed disease onset, but life span was not significantly increased. There were slight improvements in motor function [72].

Rizvanov et al. evaluated engraftment of genetically modified UCBs. In the first study, they transfected VEGF and L1 cell adhesion molecule (L1CAM) transgenes into UCBs and transplanted them by retroorbital injection. The expression of L1CAM and VEGF caused many of the cells to adopt an endothelial fate and increased engraftment into the spinal cord. These cells were localized in the walls of blood vessels, and they increased in number over 2–3 months [73]. Given that most cells in the CNS are in close proximity to the microvasculature, this approach may be useful for delivering a wide variety of neurotrophic factors. In their second study, UBCs were genetically modified to express VEGF, fibroblast growth factor 2 (FGF2), or both and were injected as before. Cells were randomly distributed through the white and gray matter 14 day post-transplantation. Nontransgenic cells expressed microglial or endothelial markers, while VEGF-FGF2-UBCs expressed S100, an astrocyte marker [74].

A Phase II clinical trial delivering umbilical cord-derived MSCs to 30 patients via intrathecal administration is currently ongoing in China (ClinicalTrials.gov ID: NCT01494480) (Table 13.1). To our knowledge, no Phase I outcomes have been published by this group.

Closing Thoughts

ALS remains a devastating disease. No currently approved therapies provide substantial improvements in life expectancy or motor function. The disease itself is highly complex, and 140 years after Charcot coined the name [75], ALS is still not well understood. A variety of biochemical abnormalities likely contribute to the demise of muscle innervation, including poor trophic support for motor neurons, mitochondrial dysfunction, and glutamate-induced cytotoxicity.

How can we address this host of problems? As we have seen, stem cell therapy is one promising approach. These cells can serve as trophic factor delivery systems, repair damaged vasculature, reduce inflammation, and dispose of excess glutamate. Many types of stem cells can slow the course of the disease, though it is not clear yet which will be the most effective in the clinic. Importantly, animal studies thus far have suggested that autologous cell transplantation will be inferior to allogeneic stem cells.

We also must not lose sight of two other important factors in animal studies. Many studies treat presymptomatic animals, which will not be the case in the clinic for the foreseeable future. In addition, the small size of rodents permits the use of extremely high cell doses relative to body size, in some cases three to five orders of magnitude higher than the doses currently being employed in clinical trials. As we base our clinical trial design on preclinical animal data, we need to give careful thought about the dose and delivery route lest stem cells suffer the same fate as recombinant neurotrophic factors.

Clinical trials of stem cells for ALS are still in their infancy. In this chapter, we have described the results of 10 Phase I clinical trials. These trials have used a variety of stem cell types and delivery methods. One major concern with the use of stem cells is tumor formation. There has been no indication of aberrant cell growth in any ALS trial published thus far; and, even with injection into the brain and spinal cord, there have been few serious adverse events. Unfortunately, there has also been little convincing evidence demonstrating efficacy, since these are primarily safety studies enrolling small numbers of patients. In addition, the placebo effect is a significant confounder in ALS trials [76]. Therefore, it is impossible to determine if the small gains observed in some studies are attributable to the therapy. There are 21 planned and/or ongoing clinical trials of stem cells for ALS, including a Phase II/III trial in Mexico (Table 13.1). However, to the best of our knowledge, none will be double-blinded, placebo-controlled studies. Therefore, it will be quite some time before we know if any of these therapies offer meaningful treatment for patients.

Of the 21 planned and ongoing trials, 16 use autologous sources of cells. Although we can appreciate the complications that arise when using allogeneic cells (graft versus host disease and graft rejection), in vitro data and in vivo rodent studies have unanimously shown that cells derived from healthy sources are superior to those derived from ALS patients or animal models of ALS. Thus, there are no preclinical data to support more than 75 % of these trials. We find this fact to be worrying. Proof-of-principle studies are needed using stem cells from ALS patients to support the use of autologous transplantation.

Although they have not yet reached the clinical trial phase, the use of genetically modified cells has shown promise in animal models. Increasing the effectiveness of each transplanted cell may help reduce the cell dose needed to treat ALS and overcome dosage concerns. iPS cells, in particular, may help bridge the chasm between autologous and allogeneic cells, allowing us to fix the mutation in the stem cells before returning them to the patient. Although we still have a long road ahead of us, stem cell-based therapies have a bright future in the treatment of ALS and other neurodegenerative diseases.

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Chapter 14
Stem Cells for Multiple Sclerosis

Pamela Sarkar and Neil Scolding

Abstract Multiple sclerosis (MS) is a common cause of progressive neurological disability, particularly affecting young adults, and no currently available therapies have any clinically meaningful impact in reversing, halting, or even slowing progression. Stem cell therapies have for several decades held out the prospect of addressing this major therapeutic challenge. Classical *cell replacement* approaches envisaged transplanting stem cells to replace lost oligodendrocytes and to remyelinate denuded axons in focal MS lesions. However, the prominent role of diffuse axonal damage in generating progressive disability limits the applicability of this strategy. A second disparate approach to stem cell therapy in MS is to use autologous *hematopoietic stem cells*, aiming to regenerate the subject's dysfunctional immune system and halt inflammatory damage. Finally, what we have termed *restorative cell therapy* aims to exploit the multiple reparative and/or disease-modifying capacities of autologous mesenchymal or other cell populations, principally from the bone marrow, but potentially from alternative tissues (such as fat), to limit and reverse tissue damage in multiple sclerosis.

Keywords Multiple sclerosis • Remyelination • Cell therapy • Neurodegeneration • Mesenchymal stem cells

Introduction

Multiple sclerosis (MS) affects some 2.5 m people worldwide, principally young adults. It carries an economic burden of around $10 billion annually in the USA and 9 billion euros across the European Union, these costs largely representing the direct and indirect consequences of progressive disability. Most patients start their disease course with a relapsing-remitting presentation, that is, with good recovery after attacks, but over 80 % of patients ultimately develop progressive disability,

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with a median time to progression of some 15 years. Immune-based treatments have proved increasingly effective in reducing relapse frequency, but none so far has been shown to have a meaningful clinical impact in reversing, halting, or even slowing progressive disability. Developing such therapies and targeting established progression is therefore a major healthcare priority. Cell therapy may offer one possible solution.

The question of stem cell therapy for multiple sclerosis has evolved considerably over the past decade and has now acquired considerable complexity. The development of *cell* therapy for MS arguably became realistic almost 40 years ago—long before the explosion of interest in stem cells—with the work of Bill Blakemore in Cambridge [1]. In a remarkable initial experiment, Blakemore showed that exogenous myelinating cells (Schwann cells in this instance), injected into demyelinated lesions in the central nervous system (CNS), achieved successful remyelination. Proof of the therapeutic principle of replacing oligodendrocytes damaged by MS disease processes with healthy (re)myelinating cells was thereby offered. Years later, the major problem of identifying the best candidate remyelinating cell type appeared to have been solved by the emergence of stem cells [2, 3], but, paradoxically, highly informative contemporaneous studies of the clinical biology of MS cast no little doubt on the underlying basis of replacement cell therapy as a treatment approach to this disease [4, 5]. At the same time, other sources and types of stem cell came into focus and alternative ways of exploiting their properties emerged. In consequence, it can now be argued that stem cell therapy in MS is thought of in three quite different ways:

- "Classical" stem cell therapy—aiming to use stem cells in a "cell replacement" strategy, to repair CNS myelin;
- Hematopoietic stem cell therapy—aiming in effect to replace or reset the subject's misfiring immune system, in order to prevent future CNS inflammation; and
- "Restorative" stem cell therapy—utilizing complex additional properties of certain stem cell types with the combined aims of both limiting nervous tissue damage and promoting endogenous tissue regeneration and repair.

These three approaches clearly have different (if overlapping) aims and make use of different types of stem cells. Partly because of this, many now prefer the term "cell therapy" to "stem cell therapy." The three strategies are at different stages of development—some nearer and some further from the clinic. All, as we hope will become clear, are important.

"Classical" Stem Cell Therapy: Replacing Lost Oligodendrocytes for Myelin Repair (Fig. 14.1)

As far as this author is aware, there has only been a single clinical experiment, not formally published [6], exploring this approach in (two) MS patients, and this studied the safety and feasibility of (autologous) Schwann cells, not stem cells.

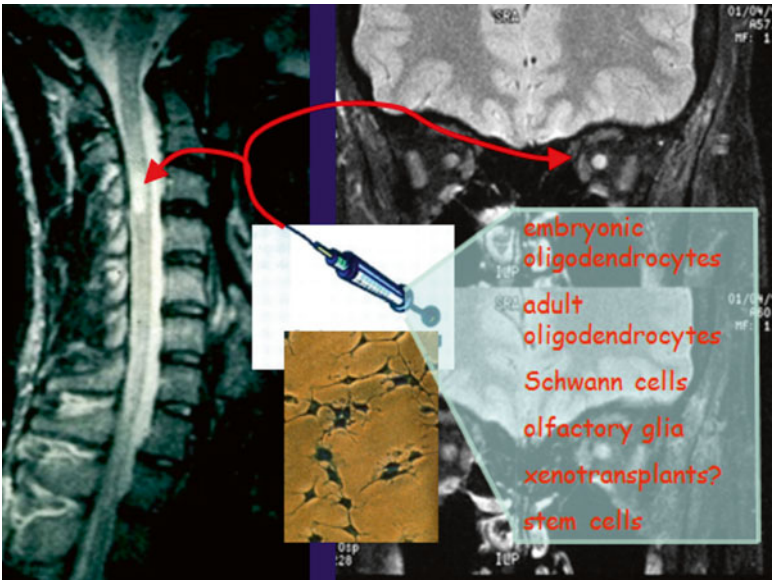

Fig. 14.1 The "classical" approach to cell therapy in MS—the direct injection of cells capable of producing myelin into MS lesions shown by MRI scanning

The initial enthusiasm for cell replacement was built on various aspects (as then understood) of MS that appeared to make this disease an ideal test bed for cell replacement therapy [7]. A single cell type was targeted by the immune system for damage, the oligodendrocyte; axons were preserved, so that remyelination should (and in rodent studies, could) restore efficient conduction and neurological function; demyelination occurred in focal patches that could readily be identified by MRI scanning and so putatively injected with remyelinating cells. And as mentioned above, rodent experiments appeared to prove the biological feasibility of this approach.

But a broader and deeper understanding of the biology of MS has cast considerable doubt on this rationale [4, 5]. Spontaneous remyelination in MS, first observed some 50 years ago and initially thought to be sparse and "abortive" [8], has been studied in great detail over the last decade in autopsy studies and found to be far more widespread and successful than hitherto thought [9, 10]—so that the underpinning need for promoting myelin repair has been questioned.

The explanation for this unexpectedly widespread spontaneous myelin repair is that endogenous oligodendrocyte progenitors are already present within MS lesions and present in significant numbers [11, 12]. There is also evidence that resident neural stem cells are likewise present and indeed that they react to disease processes by increasing their numbers [13]. The implication is that where remyelination does fail, the "problem" is more subtle than numbers of potential remyelinating cells: arrested maturation, disruption in the crucial initial interactions between axon and oligodendrocyte process, or other abnormalities of cell:cell signaling are postulated

explanations for the failure of myelin repair [14]. "Simply" adding exogenous cells appears unlikely to be the answer.

It might be asked why, if spontaneous myelin repair is so successful, the majority of MS patients develop significant disability? The answer further undermines the rationale of replacing myelinating cells as a therapy: namely that axon damage and also neuronal loss likely play a greater role than persistent demyelinated lesions in explaining progressive disability in MS [15, 16]. The disease is far from being one that exclusively damages oligodendrocytes.

And then finally, such neuro-axonal loss is now known to occur not only within focal (and MRI-disclosed) lesions, but diffusely, in gray and white matter throughout the brain and spinal cord. Focal injection of cells could do little for such diffuse damage.

That said, not all these "problems" necessarily always apply. MS also sometimes causes large demyelinating lesions which are likely to contribute to persistent disability [17]; and not all relapses are followed by complete recovery: spontaneous repair is not always sufficient. In these instances, focal injection of stem cells, aiming to supplement myelin repair, may well prove valuable. Additionally, inherited and other acquired disorders of myelin may involve lesions where permanent myelin loss is the principal cause of disability. Injecting stem cells as a source of remyelinating oligodendrocytes may still have promise in some clinical scenarios.

No less important, it is also undoubtedly true that the continuing study of remyelination biology, both stimulated and enabled by the prospect of cell therapy in MS, has proved remarkably successful in elucidating the cellular and molecular events underlying myelin repair [14]. Whilst "classical cell therapy"—injecting cells into lesions—has, as a consequence, become a somewhat less logical treatment strategy than formerly considered, insights from cell biology have offered a significant number of highly attractive molecular targets for small molecule or other drug-related therapeutic approaches specifically designed to promote and enhance spontaneous myelin repair [18, 19]. Delivered systemically, these would clearly offer the prospect of addressing more diffuse disease processes. The translation of such potential treatments from experimental to clinical studies in patients is advancing rapidly [20].

Immune Reconstitution: Hematopoietic Stem Cell Therapy

Autologous hematopoietic stem-cell transplantation (aHSCT) was originally conceived as an alternative to whole bone marrow transplantation, used to rescue patients from life-threatening bone marrow aplasia during the course of high-dose total body irradiation or myelo-ablative chemotherapy for leukemia. In multiple sclerosis (MS), a single dose of chemotherapy and/or total body irradiation is used with the aim of suppressing or preferably ablating the auto-destructive effector and memory cells of the immune system, allowing remission from MS autoimmune activity; aHSCT then offers the reconstitution of a normal (i.e., non-MS orientated)

immune and hematopoietic system [21]. This is fundamentally different from most conventional immune-modulatory or immunosuppressive regimens in aiming to restore tolerance and remove the autoimmune process, regenerating a fully functional immune system [22]. While there is little direct proof in treated MS patients that this does indeed occur, i.e., that autoimmune clones are eliminated [23], the clinical and radiological effects on inflammatory disease activity are substantial.

Impetus for the clinical translation of this approach was provided in the early 1990s by laboratory studies showing that high dose of cyclophosphamide or total body irradiation followed by syngenic bone marrow transplantation brought about complete inhibition of chronic relapsing autoimmune encephalomyelitis (CR-EAE) in the mouse, with a total inhibition of spontaneous relapses during a follow-up period of 2 months [24].

In patients, HSCs can be collected from the bone marrow by aspiration from the iliac crest or by drug-induced mobilization of peripheral blood HSC. Commonly used stem cell mobilization regimens include granulocyte-colony stimulating factor (G-CSF) administered concurrently with steroids and cyclophosphamide [21]. Prior to infusion, the collected graft can be manipulated to remove immune, auto-reactive T cells through the positive selection of $CD34^+$ cells or the negative deletion of T cells and frozen for storage while the patient undergoes "conditioning"—ablation or partial ablation of their immune system.

The most common conditioning regimen reported to European Group for Blood and Marrow Transplantation (EBMT) used to eradicate auto-reactive clones in the target organ is the BEAM regimen (carmustine, cytarabine, etoposide, and melphalan), all of which drugs can cross the blood–brain barrier [25]. Auto-reactive T cells can also be significantly depleted by the infusion of agents such as polyclonal antithymocyte globulin (ATG) or alemtuzumab. The intensity of the conditioning regimen must strike a balance between adequate immune ablation and regimen-related morbidity and mortality: safer, lower intensity regimens are increasingly explored. No single conditioning regimen has so far been shown markedly superiority to others [22]. Previous MS treatments such as interferons may affect aHSCT [22].

After completion of the conditioning regimen, the cryopreserved graft is thawed and the cells are infused. They then home to marrow space, where they seed and proliferate. Grafted cells mature into circulating blood cells and contribute to de novo lymphopoiesis.

The conditioning regimen is followed by the aplastic phase, but the graft allows recovery of the cell count some 10–20 days after infusion [26]. Expected effects from this include febrile neutropenia and infection. In the mobilizing and conditioning period, there may be relapses, and there is some suggestion that these may be associated with GCSF [26]. An engraftment syndrome consisting of noninfectious fever ± skin rash has occurred. Other late toxic effects (>100 days after transplantation) include Varicella Zoster infection and secondary autoimmune disorder (i.e., thyroiditis) [27].

The neurological outcome has been assessed in relatively small single center Phase I/II trials and in larger pooled studies [28–31]. The approach is effective in markedly reducing relapses. In relatively small studies (less than 75 patients), more

than 85 % of MS patients who received a conditioning regimen of BEAM and ATG were rendered free from clinical relapses in the absence of ongoing treatment with other disease-modifying agents [32, 33].

Some studies also reported beneficial effects on progression. For example, in one US open study of 21 patients, all were described as free from progression, and 16 were free of relapses, after a follow-up period of just over 3 years [34]. Some suggested, however, that more severely disabled patients, with a high pretransplantation disability score (EDSS > 6), were more likely to continue to deteriorate. Better outcomes were suggested for recipients younger than 40 years of age and diagnosed within the preceding 5 years [26].

It was also suggested that more "malignant" forms of MS, characterized by a rapidly evolving course with progression to severe disability, responded particularly well to aHSCT. In many reports of aHSCT in chronic MS, the reported improvement in EDSS scores was modest (0.5–1.0 range). By contrast, in patients with "malignant" MS, with follow-up extending to 4 years, EDSS scores dropped from a mean of 6.8 prior to aHSCT to a mean of 3.1 [35–37]. The inference was drawn that aHSCT might be more effective in the presence of active neuroinflammation [21].

MS patients undergoing aHSCT experienced comparable regimen-related complications to patients undergoing aHSCT for lymphoma. Urinary tract infections were common. MS patients with a greater degree of disability prior to transplantation were at risk of developing further loss of mobility due to chemotherapy-induced cachexia and myopathy. The risk of late opportunistic infections was small once immune reconstitution had occurred. Treatment-related mortality (TRM) when aHSCT was first introduced was much higher (up to 20 %) [38]. TRM during the period of 2000–2007 was reported to be significantly decreased to 1.3 % [27].

Clinical trials and observational studies continue. A recent single center experience of 123 relapsing-remitting and 28 secondary-progressive patients, with a median follow-up of 2 years, reported a 4-year relapse-free survival of 80 % and progression-free survival of 87 %. Importantly, post hoc analysis showed that disability (measured by EDSS) did not improve significantly in patients with secondary-progressive MS or in those with a disease duration of over 10 years [39]. Again, this would be consistent with the observed reduction in disability being at least partially explained by recovery from relapse. Indeed, one interpretation was that this study helped show that, while there was a clear, potent and lasting effect in suppressing relapses, "autologous HSCT does not appear to be effective against established progressive forms of MS and, absent new data, additional trials of these protocols are probably not indicated for patients with progressive MS" [23]. An additional complexity to interpreting these studies is that alemtuzumab was used in many conditioning regimens and does itself cause a substantial and sustained reduction in relapse rate. A further recent multicenter Phase II study, again including both relapsing-remitting and secondary progressive patients, also showed a substantial reduction in inflammatory disease activity and relapse frequency, but reported no effect on the progression of disability [40].

A joint EBMT and Centre for International Blood and Marrow Transplant Research (CIBMTR) registry-based, long-term follow-up study and a proposed

Phase III randomized trial of stem cell transplants versus best available therapy for patients with highly active MS who failed interferon-beta therapy will provide further information regarding outcome and benefit of aHSCT [41].

"Restorative" Cell Therapy

The classical properties of the stem cell are self-renewal and the ability to differentiate into multiple specialized cell types. It was these properties that first projected stem cells forward as a solution to the question of the ideal cell type to use to replace damaged oligodendrocytes in early cell therapy approaches to MS [7]. But it has become clear over the past decade or more that many stem cell types have additional potentially beneficial properties, unrelated to forming specialized cells. In some situations and with certain specific stem cell types, these "noncanonical" properties, some paracrine, others not, may play a considerably greater role in any therapeutic effect than conventional differentiation and cell replacement [42].

In relation to neurological disease, both neural stem cells and mesenchymal stem cells, the latter derived mainly from bone marrow (though in some studies also from other tissues, including adipose tissue) have been shown in experimental studies to have therapeutic potential that depends on such noncanonical properties [43–45]. Bone marrow-derived mesenchymal stem cells (Fig. 14.2)—which most authorities consider to have no capacity at all for differentiating into myelin-forming oligodendrocytes—have attracted probably the most attention.

Bone marrow has long been known to contain hematopoietic stem cells. But various other stem-cell types are also present, including mesenchymal stem cells (though there are others, and mesenchymal stem cells themselves are a heterogeneous population [46]). Furthermore, though first identified in bone marrow, mesenchymal stem cells are present in many tissues—indeed, in every tissue in which they

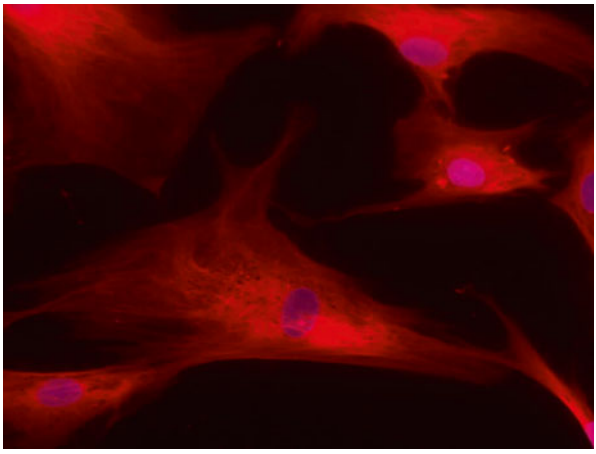

Fig. 14.2 Human mesenchymal cells growing in cell culture: cells with a wide range of potentially therapeutic properties [83]

have been sought [46, 47]. Their normal function within the bone marrow is to do with maintenance of the hematopoietic stem cell niche, but in addition there has been increasing evidence that mesenchymal stem cells have systemic activities to do with tissue repair. They may achieve such a function through multiple mechanisms, and many of these are relevant to MS and offer the prospect of ameliorating a number of various pathological processes now known collectively to contribute to the development of tissue damage in MS [42, 48]—what we have termed *restorative* cell therapy.

Remyelination

Studies commencing 15 years ago in experimental animals with nonimmune demyelination showed that not only isolated mesenchymal stem cells but also mixed populations of unseparated, nonexpanded bone marrow cells promote myelin repair following intravenous injection [49, 50]. The mode of action was not clear. Intravenously delivered bone marrow-derived cells successfully infiltrate the brain and spinal cord, inflamed or otherwise [51, 52], and they proliferate and migrate toward cytokines expressed in multiple sclerosis lesions [53]. Initially it was considered that bone marrow-derived cells arriving in demyelinated lesions might differentiate into Schwann cells and lay down peripheral-type myelin. Current thought, however, centers on the later-discovered ability of mesenchymal stem cells to interact with and stimulate local CNS endogenous neural precursors, encouraging both their proliferation [54], and their directed differentiation into oligodendrocytes [55]. Mesenchymal stem cells also secrete trophic factors for oligodendrocytes [56] which might additionally promote remyelination.

It is also reported that mesenchymal stem cells reduce gliotic scar formation in the CNS [57], gliosis being widely considered a major impediment to spontaneous myelin repair. They can also promote new blood vessel growth, and this too would also be expected to enhance tissue repair [58].

Suppressing Inflammation, Modulating Immunity

Bone marrow-derived cells have pronounced immune-modulating properties [59], affecting both innate and adaptive immune systems. Unsurprisingly, therefore, clinical effects in many systemic autoimmune diseases have been sought and in some cases benefit has been reported [60]. In relation to multiple sclerosis, numerous studies have shown both mesenchymal stem cells and mixed populations of bone marrow-derived cells successfully to abrogate various experimental allergic encephalomyelitis models through increasingly well-delineated immunosuppressive actions. Some authorities consider these immune effects sufficiently potent to justify clinical testing in relapsing-remitting MS [61] (MESEMS; *ClinicalTrials.gov Identifier* NCT01854957), almost irrespective of these cells' putative reparative or regenerative effects.

Neuroprotection

What of the progressive loss of axons and neurons in multiple sclerosis that contributes so greatly to the relentless accumulation of disability? Considering the enormous structural complexity of neuronal pathways, neuronal cell replacement approaches for brain repair remain extremely hard even to imagine for the foreseeable future. Reestablishing normal synaptic pathways in the developed CNS, capable of restoring function, appears a very remote prospect. The emphasis at present therefore remains on developing approaches to limit and reduce such damage and/or to ameliorate its consequences.

In multiple sclerosis, axon damage and neural cell loss likely result from several mechanisms. Inflammatory and immune mediators, possibly "sequestered" within the CNS, contribute [62], and so the immunomodulating/suppressing properties, both local and systemic, of bone marrow-derived cells are relevant and may potentially benefit. Mesenchymal stem cells reduce axon loss in various immune-mediated EAE models [63].

But they also help reduce axon damage in nonimmune CNS injury, including for example, experimental stroke models [64]. Here, other beneficial properties of these cells are more relevant. Human mesenchymal stem cells release superoxide dismutase-3 (SOD-3), with powerful neuroprotective effects [65]—and damage from reactive oxygen radicals is also postulated to occur in multiple sclerosis [66]. A range of neurotrophic factors, all constitutively synthesized, also contributes to these cells' neuroprotective properties. Mesenchymal stem cells also promote CNS neurite outgrowth and remodeling [67].

It is important to mention that adipose stromal cells likewise exhibit neuroprotective properties [68].

Cell Fusion

A particularly intriguing additional property of BMDCs has recently emerged, and this is cell fusion. Bone marrow-derived cells have long been known to fuse with certain differentiated cell types. The physiological significance of such fusion is, as yet, uncertain, but it appears quite clearly to occur in vivo as well as in vitro and can involve CNS neuronal cell types as "partner" cells [69]. Experimentally, local or systemic inflammation or immune activation promotes the fusion of circulating bone marrow cells with neurons following infiltration of the CNS, and this is seen with both rodent and also with human mesenchymal stem cells [70].

Fusion appears to represent a neuroprotective process by which healthy nuclei or functional genes from the mesenchymal stem cell are introduced into degenerating cells, helping to restore or rescue damaged neurons [71]. Rather extraordinarily, mesenchymal stem cells can also protect tissue by directly transferring mitochondria to vulnerable cells [72], membrane fusion (likely relating to nanotube formation or exosome transfer) representing the underlying mechanism common to both cell fusion and mitochondrial "donation." Preliminary evidence has emerged

that fusion of infiltrating (endogenous) bone marrow-derived cells with Purkinje cells, with subsequent heterokaryon formation, occurs spontaneously in MS patients [73].

Diffuse Damage

What of the question of multiple sclerosis as a nonlesional disease and the more diffuse gray matter disease and atrophy that form the key substrates of sustained disability in MS? Injecting cells into specific lesions could offer but little prospect of benefiting this aspect of the pathophysiology, but a cell therapy delivered systemically, rather like any conventional drug therapy, may well have more rationale—as well as being safer than a neurosurgical procedure.

Following intravenous injection, many cells are trapped in the lungs, but significant numbers still clearly enter the CNS and become widely distributed—not only in experimental models but in human subjects too [74], offering the clear possibility of a therapeutic effect where it is required. (Additionally, even cells "trapped" in the lungs may indirectly exert clinically relevant systemic anti-inflammatory therapeutic effects, clearly an intriguing area of future research.)

Others have explored delivery of bone marrow-derived cells in patients with neurological disease using injection into the carotid arteries (in multiple system atrophy, though not, as far as we are aware, in multiple sclerosis) [75]. Whilst appearing clinically safe, and while a higher proportion of injected cells would be expected to enter the CNS, there are indications that potentially hazardous microemboli form within the cerebral arterial system using this approach, which has constrained enthusiasm.

Clinical Translation

As with hematopoietic stem cell translation, restorative cell therapy using other bone marrow-derived cells delivered intravenously to exploit their reparative and neuroprotective effects has also begun the journey from laboratory to clinic—though only in more recent years so that published trials thus far are fewer and smaller. (The same is not necessarily the case in the clinical exploration of bone marrow-derived cells in other diseases: *ClinicalTrials.gov* currently lists around 2000 trials studying bone marrow-derived cells; and in myocardial infarction, both large scale Phase III randomized controlled trials and meta-analyses of trials are now reported.)

Various groups have published small safety and feasibility studies exploring autologous bone marrow-derived cell therapy in chronic multiple sclerosis, some using mixed/unseparated cells, others purified and expanded mesenchymal stem cells [76–80]. Most have utilized intravenous delivery, but intrathecal injection has

also been explored. The results have generally confirmed the safety and feasibility (though a transient meningeal syndrome is reported with intrathecal delivery); and some have reported preliminary and uncontrolled evidence from detailed neurophysiological studies of beneficial effects [77, 79]. Larger, controlled Phase II studies are now underway [81].

Conclusion

It is hopefully clear that, while the subject of stem cell therapy—or cell therapy—in multiple sclerosis has become increasingly complex and multifarious, this evolution has been a positive response to our rapidly advancing knowledge on the one hand of the underlying clinical biology of multiple sclerosis and on the other of stem cells and their various properties and types. It is hard to predict what this topic will look like in a decade or two. It has been suggested that many forms of cell therapy are no more than necessary stepping stones on a pathway that will rapidly see them replaced by more sophisticated forms of molecular therapy. We have already seen how the biological knowledge emerging from studies of classical oligodendrocyte replacement therapy have yielded therapeutic trials of small molecules designed to promote remyelination. Similarly, restorative cell therapy might, it is suggested, be successfully mimicked (obviating the need for cell harvest and preparation) by molecular therapies aiming to stimulate the release of specific cell populations from the bone marrow into the circulation, or by agents that enhance the migration of bone marrow derived cells from the circulation into neural tissue. What such approaches are unlikely, however, to reproduce, is what might be called the "afferent" side to cell therapy, a subject we have not touched upon. It is increasingly clear that the multiple potentially therapeutic capacities of which some bone marrow-derived populations are capable are not randomly activated in all disease situations: this is no "shot gun" effect. Rather, infiltrating cells sense and react: specific pathways are triggered in different tissues and in response to different forms of tissue damage and different disease processes [82]. It would be challenging to reproduce this by administering molecules rather than cells. There is as yet much mileage in the experimental and clinical exploration of cell therapy in relation to multiple sclerosis and indeed many other neurological diseases.

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Chapter 15
Cell Therapy for Pediatric Disorders of Glia

M. Joana Osorio and Steven A. Goldman

Abstract The childhood disorders of glia comprise a group of diseases that include the pediatric leukodystrophies and lysosomal storage disorders, cerebral palsies and perinatal hypoxic ischemic encephalopathies, and selected neurodevelopmental disorders of glial origin. Essentially, all of these disorders are associated with oligodendrocytic failure and dysmyelination, whether congenital or progressive, and most are attended by astrocytic dysfunction as well. In light of the wide range of disorders to which glial dysfunction and demyelination may contribute, and the relative homogeneity of glial progenitor cells (GPCs) and their derivatives, the glial disorders may be uniquely attractive targets for cell-based therapeutic strategies, and the pediatric disorders especially so. As a result, GPCs, which can distribute throughout the neuraxis and give rise to new astrocytes and myelinogenic oligodendrocytes, have become of great interest as candidates for the therapeutic restoration of normal glial architecture and function, as well as new myelin, to the pediatric brain.

Keywords Stem cells • Pluripotential stem cells • Neural stem cells • Glial progenitor cells • Oligodendrocyte progenitor cells • Myelin • Remyelination • Leukodystrophy • Mouse models

Childhood disorders of glia, including astrocytes as well as oligodendrocytes and their associated myelin, include a broad spectrum of etiologies and pathology [1]. They can be hereditary, with identified allelic mutations and well-characterized genetic defects, as in the lysosomal storage disorders and the inherited hypomyelinating

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disorders like Pelizaeus-Merzbacher disease (PMD). Dysmyelinating disorders may also be acquired, as occurs in the periventricular leukomalacias associated with prematurity, germinal matrix hemorrhage, and perinatal hypoxic ischemic injury. In addition, a group of well-described yet poorly understood neurodevelopmental cognitive disorders appear to include dysmyelination as part of their primary pathology; these include autism spectrum disorders and juvenile onset schizophrenias, among others. In light of the significant contribution of myelin pathology to all of these disorders, cell-based strategies intended to promote remyelination may present therapeutic opportunities for this entire group of largely pediatric maladies. In this chapter, we will focus on the development of cell-based treatment for the congenital disorders of glia, and in particular for diseases of myelin. Importantly, while cell-based therapeutics intended for use in the central nervous system have been developed for a variety of reasons, including as anti-inflammatory agents and vehicles for trophic support [2], we will focus here solely on their potential use in cell replacement following glial and myelin loss, for the purpose of structural reconstitution of the brain's white matter.

Cell Types for Cell Replacement

Neural Stem Cells Neural stem cells (NSCs) and their derived glial progenitor cells (GPCs) have been the most widely assessed phenotypes for cell replacement in the diseased or injured brain. NSCs are the self-renewing and multi-lineage competent derivatives of the early neuroepithelium [3, 4], whose progeny can include all three major neural phenotypes, including neurons, astrocytes, and oligodendrocytes. In postnatal humans, NSCs persist in the subependymal zone and hippocampus [5–8], but their numbers in the adult brain are relatively sparse [8]. Nonetheless, they can be isolated and purified from adult [8–11] as well as fetal human brain [12, 13] and readily expand in vitro. CD133/proponin^{+}-defined NSCs, and in particular their CD24$^{-/lo}$ fraction, can grow as neurospheres, and differentiate largely as neurons and astrocytes in vitro, yet remain myelinogenic in vivo [13].

Upon transplantation, NSCs can generate neurons and glia in a context dependent fashion, including oligodendrocytes in the white matter, and as such can be used to restore myelin in myelin-deficient brain regions [14]. As a result, these cells have been used as myelinogenic vectors in rodents with spinal cord injury, in models of both acute and progressive myelin loss, and most recently in hypomyelinated shiverer mice, in which transplanted NSCs generated functional oligodendrocytes and abundant compact myelin [15]. Indeed, NSCs have already been used in clinical trials to assess their safety in spinal cord injury (NCT01725880, NCT02163876, NCT01321333), Batten disease (NCT00337636) [16], PMD (NCT01005004) [17], and amyotrophic lateral sclerosis (NCT01730716) [18].

Glial Progenitor Cells While NSCs can be deployed to treat disorders of myelin, their progeny are primarily astrocytes and neurons, and their production of oligodendrocytes after transplantation is variable and context-dependent. Their in vivo differentiation is thus difficult to instruct, allowing the potential for both heterotopic neuronal differentiation and astrocytosis; as such, they are inefficient as vectors for

focused oligodendrocytic and astrocytic production. In contrast, GPCs comprise an already lineage-restricted glial progenitor population, that may be better suited to treating the disorders of glia, and more appropriate for myelin disease in particular [19]—although as a practical matter, GPCs lack the sustained mitotic competence and scalability of NSCs.

GPCs arise from NSCs in the subventricular zone and migrate with development to populate both the subcortical white matter and cortical gray matter. They comprise 3–5 % of all cells in the adult brain [20, 21] and can give rise to oligodendrocytes and astrocytes. As the sole source of new oligodendrocytes in the adult brain, they are the principal remyelinating cell type of the adult CNS [22, 23]. While glial progenitors have thus also been called oligodendrocyte progenitor cells (OPCs), human glial progenitors appear to remain bipotential until their last division, so that the two terms, GPCs and OPCs, are functionally synonymous. For consistency's sake, in this chapter we will designate these cells as GPCs.

While the presence of GPCs in the adult human brain was inferred in several early studies that identified immature oligodendroglia in adult brain tissue [24, 25], mitotic bipotential GPCs were first isolated from the adult human by transfecting dissociated human subcortical white matter with hGFP placed under the control of the promoter for oligodendrocytic cyclic nucleotide phosphodiesterase (P/hCNP2), one of the earliest proteins to be synthesized by lineage-restricted oligodendrocytes [20]. The GFP^+ cells were mitotic, initially expressed gangliosides recognized by the monoclonal antibody A2B5, and matured as oligodendrocytes, progressing through a stereotypic sequence of A2B5, O4/sulfatide, and O1/galactocerebroside expression [26]. Of note, this study highlighted the utility of A2B5 as identifying mitotically competent human GPCs, while confirming earlier observations that the O4 antibody against sulfatide, commonly used to identify GPCs in rodents, recognized only postmitotic oligodendroglia, and not their mitotic progenitors, in humans [24, 25].

Remarkably, when removed to low-density, high-purity culture, single adult human GPCs were revealed to be multipotential in nature and able to give rise to neurons as well as astrocytes and oligodendrocytes [27]. This study mirrored similar observations in rodents [28], which had also suggested the intrinsic multilineage competence of adult parenchymal GPCs, and their in vivo fate restriction. Indeed, while adult human GPCs gave rise to both glial cells and neurons in vitro, they were primarily committed to glial fate in vivo, generating oligodendrocytes and astrocytes within the white matter [27, 29, 30], and yet they could still generate neurons if introduced directly into neurogenic environments such as the prenatal olfactory stream and hippocampus. Together, these data established that the local environment plays a strong role in the fate determination of transplanted GPCs [27, 31].

Fetal Glial Progenitors To derive a more accessible and scalable source of transplantable cells, GPCs were later purified from the fetal forebrain using fluorescence activated cell sorting (FACS) to select $A2B5^+/PSANCAM^-$ cells, that expressed the early glial progenitor marker A2B5 and were depleted of immature N-CAM-defined neurons [29]. These fetal human GPCs co-expressed the NG2-chondroitin sulfate proteoglycan [32] as well as the PDGFRα receptor [33]. While PDGFRα expression is selective to GPCs, other cells, such as pericytes, also express

NG2. Thus, to obtain GPCs more selectively, we isolated cells using an antibody against the PDGFα receptor epitope recognized as CD140a. These cells migrated broadly, yet differentiated and myelinated more rapidly than fetal A2B5^{+}/PSA-NCAM^{-} GPCs, of which they were largely a subset, and as such proved to be a more efficient therapeutic vector [34].

Functionally, fetal and adult GPCs are readily distinguished from one another. When transplanted into the hypomyelinated shiverer mouse, fetal GPCs show rapid and extensive migration and engraftment, and generate abundant astrocytes and oligodendrocytes alike, the latter of which mature and myelinate only slowly. In contrast, adult-derived GPCs migrate less, but mature and myelinate more rapidly and efficiently, with little in vivo astrocytic co-production [29].

Pluripotential Stem Cells Pluripotential cells, which in broad terms include embryonic stem cells and induced pluripotential cells, have a major advantage over tissue-derived cells, in that they offer a readily scalable source of cells that can be expanded and differentiated to different types. Oligodendrocytes and their immediate precursors, GPCs, were first generated from human ES cells [35] and subsequently from iPSCs [46]. In both cases, the preparation protocols include the serial application of agents intended to replicate the environment experienced by developing cells along the oligodendroglial lineage (reviewed in [53]).

Induced pluripotent stem cells (iPSCs) are somatic cells genetically reprogrammed to a pluripotential stem cell state. The first successful reprogramming strategies utilized retroviral delivery system to express four transcription factors (Oct4, Sox2, Klf4, and c-myc) in mouse fibroblasts [36, 37] and later in human cells [38, 39]. Given the inherent risk for insertional mutagenesis, aberrations in epigenetic reconfiguration, and the risk of oncogenesis if reprogramming genes are incompletely silenced after differentiation [40–42], later studies focused on minimizing or excising residual transgene footprints, so as to create a product more appropriate for cell replacement therapy. The first generation transgene-free iPSCs were generated by Cre-mediated recombination using fibroblasts derived from patients with Parkinson disease [43]. The same method of transgene excision was applied to generate iPSC-derived neural cells that differentiated efficiently into neurons, oligodendrocytes, and astrocytes with appropriate migration and no teratoma formation when grafted in the normal rat [44]. Thereafter, successive generations of "footprint-free" strategies were developed using transient transfection with episomal plasmids or minicircles, infection with Sendai virus, transduction with synthetic mRNA and miRNAs, and use of the piggyback transposon (reviewed in [45]).

Human iPSCs can be differentiated to glial fate following similar protocols to those developed for use with hES cells [46]. Differentiation to oligodendrocyte precursor cells (GPCs) was initially demonstrated in rodent iPSCs [47, 48] and later in human iPSCs [46, 49]. Transplantation of human iPSCs-derived GPCs resulted in differentiation as myelin-producing cells in vivo, both focally, after transplantation in lysolecithin-induced demyelinated rat optic chiasm [49] and more broadly

throughout the neuraxis, as in the congenitally demyelinated shiverer mouse [46]. As an alternative to the use of somatic cells in generating iPSCs before differentiating to glial fate, several groups have developed strategies for the direct induction of glial phenotype from fibroblasts [50–52]. These protocols effectively skip the pluripotential stem cell stage, and thus greatly accelerate the induction process, by transducing the somatic cells with a combination of transcription factors that reprogram the recipient cells directly to oligodendroglial fate. While this strategy has not yet been successfully applied to human somatic cells, one may anticipate that the accomplishment of that step will enable new therapeutic possibilities as well, especially in those clinical settings requiring the rapid production of myelinogenic oligodendroglia from autologous sources.

Barriers to the clinical use of ES cells and iPSCs include their potential for undifferentiated expansion and tumorigenesis, as well as the need for immunosuppression to avoid rejection of allografted cells [53–55]. In addition, the need for feeder-free and xenogen-free cell culture systems in which cells can be both produced and expanded, and the consequent need for facilities compliant with the standards of good manufacturing practice (GMP), may delimit and slow the clinical adoption of these technologies.

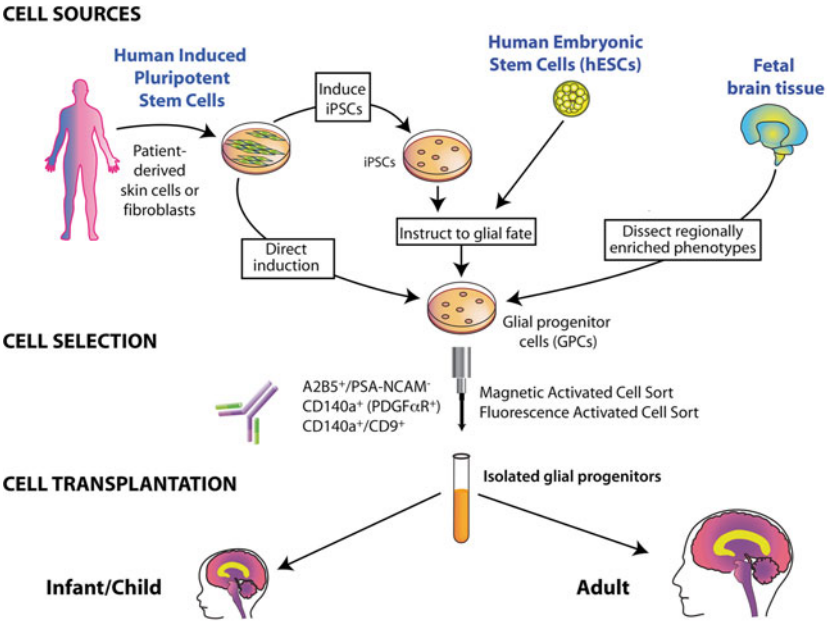
Mesenchymal Stem Cells Other cell types besides those of the CNS have been proposed as vectors for brain repair, including neural crest derivatives such as olfactory ensheathing cells and Schwann cells, and non-neural phenotypes such as mesenchymal cells. In particular, mesenchymal stem cells (MSCs), stromal cells that can be isolated from bone marrow and cord blood and are able to differentiate along a variety of mesodermal lineages, have been proposed as potential therapeutic agents for a wide variety of neural disorders. Yet the principal mechanism of action for MSCs in treating neurodevelopmental and myelin disorders appears to lie in their anti-inflammatory function and not in any ability to effect structural repair. Systemically administered MSCs appear to enter the perivascular spaces of the brain parenchyma and differentiate therein as tissue macrophages and resident microglial cells; in that capacity, they may then modulate central immune surveillance [56]. Yet beyond their microglial potential, there is little clear evidence that MSCs can differentiate as neural or macroglial cells in vivo. As such, these cells cannot be considered as appropriate vectors for cell replacement and tissue regeneration. Rather, as in the case of lysosomal disorders, these cells may act as vehicles for delivery of defective or deficient enzymes, which may be delivered to deficient host cells by mechanisms such as the mannose-6-phosphate pathway [57], by which lysosomal enzymes released from wild-type donor cells may be transported to enzyme-deficient neighbors, permitting local correction of disease-specific substrate depositions and metabolic disturbances. As such, both hematopoietic and MSC transplants have proven effective in some systemic disorders of substrate misaccumulation, such as the mucopolysaccharidoses [58, 59], though these transplants have proven of less benefit to the CNS of patients with neurological involvement.

Disease Targets for Glial Cell Therapy

Central neurological disorders comprise difficult targets for cell-based therapeutics, because of the panoply of cell types often lost in CNS disease, and the complex paracrine relationships among them. As a result, disorders most suited for cell therapy will thus involve only one or a few discrete cell types and will have well-understood etiology and pathophysiology. Among those, the glial disorders, which include the congenital disorders of myelin, are especially attractive targets (see Fig. 15.1). These typically result from pathology limited to oligodendrocytes and astrocytes, and the genetic etiology has been defined in most. In contrast, progressive disorders that affect both neurons and glia, such as neuronal ceroid lipofuscinosis (NCL), or those primarily affecting neurons, such as spinal muscular atrophy, comprise more difficult targets, given the multiplicity of cell types involved by the disease process, and the difficulty of restoring neurons and their networks to their premorbid state. In that regard, more static neurodevelopmental disorders, such as autism and neonatal brain injury, carry a different set of challenges, in that the aberrant neural network that has already been established may be resistant to structural modification.

Congenital Disorders of White Matter GPCs have been assessed as potential therapeutic vectors in a variety of diseases with prominent glial involvement, particularly in congenitally hypomyelinating disorders and lysosomal storage disorders predominantly affecting myelinating cells. More broadly, any disorder characterized by the predominant destruction of white matter may prove an appropriate target for cell replacement. The hereditary disorders of myelin are all in this category and may be considered as three principal groups: (1) the hypomyelinating disorders, characterized by decreased or absence formation of myelin; (2) the metabolic demyelinating disorders, including the lysosomal storage disorders, often characterized by deposition and misaccumulation of lipid components of myelin; and (3) those disorders resulting from gross tissue loss, such as periventricular leukomalacia [1]. In addition, some primary disorders of astrocytes, such as Alexander's disease, are associated with dysmyelination, indicating the astrocytic dependence of oligodendrocytes. In all of these, cell-based treatment strategies aim to replace the abnormal glial cells with oligodendrocytes capable of forming functional myelin, as well as to serve as vehicles for the delivery of wild-type enzymes that may be deficient in the host, and whose replacement might be sufficient to restore or maintain normal myelin by the deficient host cells.

Hypomyelinating Disorders PMD is an X-linked hereditary disorder of myelin formation caused by mutations in the PLP1 gene that encodes the proteolipid protein of myelinating oligodendroglia [60]. The phenotype of affected patients is a result of decreased or abnormally formed PLP protein and leads to significant mortality and morbidity. PMD is an attractive target for cell therapy because one particular cell type is affected, myelinating oligodendrocytes, and able to be replaced by progenitors capable of differentiation and generation of functional myelin. Similarly, other hypomyelinating disorders can be targets for cell therapy. However, some hypomyelinating disorders course with other abnormalities, and caution



Hereditary Leukodystrophies
- Congenital dysmyelination
  - Pelizaeus-Merzbacher Disease
- Lysosomal storage diseases
  - Tay-Sachs and Sandhoff's gangliosidoses
  - Krabbe's Disease
  - Metachromatic leukodystrophy
  - Mucopolysaccharidoses
  - Niemann-Pick A
- Non-lysosomal diseases
  - Adrenoleukodystrophy
  - Canavan's disease
  - Vanishing White Matter Disease
  - Alexander Disease

Cerebral Palsy
- Periventricular leukomalacia
- Spastic diplegias of prematurity
- Hypoxic-ischemic encephalopathy

Neurocognitive developmental disorders

Autoimmune Demyelination
- Multiple Sclerosis
- Neuromyelitis optica
- Transverse myelitis
- Optic neuritis

Vascular Leukoencephalopathies
- Subcortical Stroke
- Diabetic leukoencephalopathy
- Hypertensive leukoencephalopathy
- Age-related white matter disease
- Spinal cord injury

Inflammatory Demyelination
- Radiation injury

Neurodegenerative disorders

Multisystem atrophy

Huntington's disease

Fig. 15.1 Glial progenitor cell sources, phenotypes, and clinical targets. GPCs may be directly sorted from tissue, or generated from either human embryonic stem cells (hESCs) or induced pluripotential cells (hiPSCs), and then immunoselected based on their expression of either the A2B5 epitope or CD140a/PDGFαR. The CD140a phenotype includes all potential oligodendrocytes, while the tetraspanin CD9 identifies a pro-oligodendrocytic fraction [34]. The choice of tissue-, hESC-, or iPSC-derived GPCs depends upon whether allogeneic or autologous grafts are desired. Whereas autologous grafts of iPSC-derived GPCs might obviate the need for immunosuppression, their generation may take months, and their use in the hereditary leukodystrophies would first require correction of the underlying genetic disorder in the donor cell pool. Adapted from [19]

should be made because pure glial grafts may not resolve concurrent symptoms caused by other mechanisms. Among those is 18q⁻ syndrome, caused by a deletion that includes but is not restricted to the locus for the myelin basic protein (MBP) (18q22–23), and characterized by dysmorphic features, hypotonia, cognitive impairment, seizures, nystagmus, and extrapyramidal symptoms [61].

Other hypomyelinating disorders, including the PMD variant hereditary spastic paraparesis type 2, the PMD-like disorder associated with connexin 47 mutation, the broadly hypomyelinated 18q⁻ syndrome, the Allen–Herdon–Dudley syndrome, and others, may also be considered viable candidates for oligodendrocyte replacement therapy. That said, several of these disorders are not well characterized, and may exhibit either PNS or visceral organ involvement, so that careful assessment of each disease and each patient will need to be performed before initiating cell therapy.

Metabolic Demyelinating Disorders These disorders differ from the primary hypomyelinating disorders by the presence of an enzymatic deficit that leads to accumulation of abnormal metabolites that become toxic to glial cells and/or neurons. The aims of cell-based therapy for such disorders are to replace the enzymatic deficit, either by allogeneic transplantation of glial cells or by autologous transplantation of genetically modified cells expressing normal or supra-levels of the defective enzyme.

Approaches to Cell-Based Therapy

The most widely used model of congenital hypomyelination is the shiverer mouse, a spontaneous mutant deficient in MBP by virtue of a premature stop codon in the MBP gene that results in the omission of its last five exons [62]. Homozygous mice fail to form compact myelin, develop ataxia and seizures, and die by 20–22 weeks of age. Although the shiverer mouse replicates no known human disease, except for rare families with mutations in the MBP gene, in association with 18q⁻ syndrome, it provides a platform by which to evaluate the efficacy of transplanted cells in myelinating host axons in hypomyelinated environments. Context-dependent NSC differentiation into myelinating oligodendrocytes was first reported in the shiverer brain by Snyder and colleagues, using an immortalized line [14], and later by Schwartz and colleagues using epidermal growth factor (EGF)-responsive NSCs [63]; similar observations of myelinogenesis by implanted human NSCs were most recently reported by Back and colleagues [15]. Yet NSCs predominantly differentiate as astrocytes and neurons in vivo, and their efficiency of oligodendrocyte production is variable. To address this issue, we and others have focused on the use of glial progenitors as sources of myelin-producing oligodendroglia.

Mitotic human GPCs were first isolated using a CNP2 promoter-directed strategy, which permitted the identification of A2B5 as an appropriate antibody for the recognition and isolation of these cells [20]. These cells proved highly myelinogenic in vivo, as their transplantation into neonatal shiverer mice resulted in significant migration, whole neuraxis myelination of the engrafted hosts, and functional

competence of the remyelinated areas [29]. Most remarkably, A2B5-isolated fetal human GPCs proved capable of rescuing otherwise lethal myelin deficiency of the shiverer mouse, when provided as multi-site neonatal grafts [64] (see Fig. 15.2). Subsequent studies focused on isolating human GPCs on the basis of their expression of CD140a, an ectodomain epitope of the human PDGFα receptor, which is expressed by the oligodendrocyte-competent fraction of the A2B5-defined progenitor pool [33, 34]. When transplanted to the shiverer brain, these cells exhibited more rapid migration and robust myelination than the A2B5 fraction from which they were largely derived, suggesting their superiority as therapeutic vectors [34].

Subsequently, to establish a source of autologous cells able to be readily amplified for translational use, a protocol was developed to generate GPCs from human iPSCs. The cells efficiently differentiated as myelinating oligodendrocytes and astrocytes, both in vitro and in vivo, upon transplantation in the shiverer mouse brain [46]. Importantly, the transplanted shiverers lived significantly longer than their untransplanted mice, many surviving to achieve normal lifespans. No tumors were observed up to 9 months after graft transplantation. This study showed that iPSCs-derived grafts could be used in the treatment of myelin disorders.

These preclinical studies, which as a group demonstrated the ability of human GPCs to rescue congenital hypomyelination in mice [19, 46, 64], provided the basis for the first phase 1 clinical trial in PMD, that consisted of intracerebral transplantation of human NSCs into four patients with connatal PMD (NCT01005004) [17]. Patients received immunosuppressive therapy for 9 months. The trial investigators reported a favorable safety profile at 1 year after transplantation, by both clinical and radiological evaluation, but the efficacy of NSC grafts in these patients requires further evaluation with time. Long-term follow-up safety and preliminary efficacy outcomes, including clinical neurodevelopmental assessment, radiological evaluation, and neurophysiological evaluation (electroencephalogram and somatosensory evoked potentials) will be assessed 4 years after transplantation (NCT01391637).

Neural stem and Progenitor Cell-Based Treatment of Enzymatic Disorders

Neural and GPCs can act as vehicles for enzyme delivery, since wild-type lysosomal enzymes may be released by donor cells and integrated into the deficient host cells through the mannose-6-phosphate receptor pathway [57]. A relatively low number of donor cells may provide sufficient enzyme levels to restore enzymatic function, as most lysosomal enzymes are secreted at low levels and the secreted enzyme is taken up by neighboring cells and targeted to the lysosome [65]. The first study showing the benefit of NSCs transplantation in lysosomal disorders was for mucopoly saccharidosis VII (Sly disease). Transduced murine NSCs expressing beta-glucuronidase, and later human NSCs derived from fetal brain [66, 67], were transplanted in the ventricles of neonatal affected mice, resulting in restored enzymatic function [68]. In GM2 gangliosidosis (Tay-Sachs and Sandhoff disease), NSCs

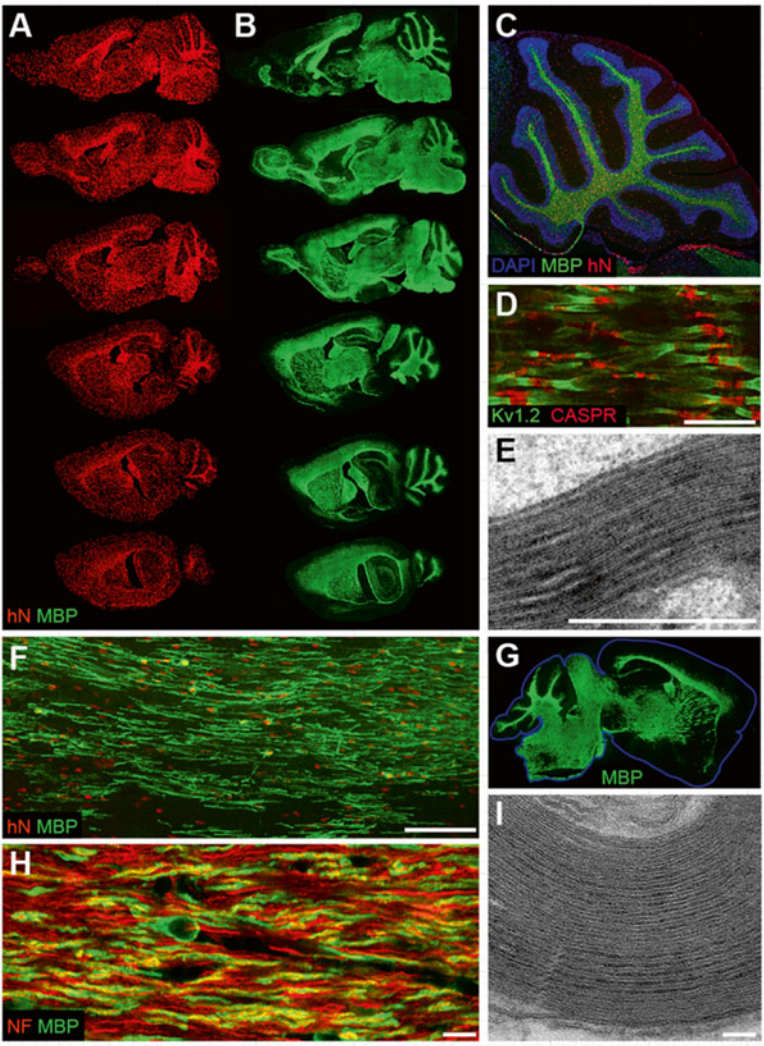


Fig. 15.2 Glial progenitor cell graft-mediated myelination of a dysmyelinated host. (**a**–**e**) Myelination of congenitally hypomyelinated shiverer mice by human fetal tissue-derived glial progenitor cells. (**a**–**b**). Representative sagittal images of an engrafted shi/shi × rag2$^{-/-}$ brain, sacrificed at 1 year of age. Each image represents a composited montage of 10× images. (**a**) Human donor cells identified by an anti-human nuclear antibody (hN; *red*). (**b**) Donor-derived myelin basic protein (MBP; *green*) in sections adjacent or nearly so to matched sections in (**a**). All major white matter tracts, including those of the corpus callosum, capsules, striatum, fimbria, cerebellum, and brainstem heavily express MBP (which is all necessarily donor derived in MBP-null shiverer mice). (**c**) Sagittal view through cerebellum of a year-old engrafted shi/shi × rag2$^{-/-}$ brain. All cells were stained with DAPI (*blue*); donor cells were identified by human nuclear antigen (hN, *red*) and donor-derived myelin by MBP (*green*). (**d**) Reconstituted nodes of Ranvier in the cervical spinal cord of a transplanted and rescued 1-year-old shi/shi × rag2$^{-/-}$ mouse, showing paranodal Caspr protein and juxtaparanodal potassium channel Kv1.2, symmetrically flanking each node. Untransplanted shiverer brains do not have organized nodes of Ranvier and, hence, cannot support

transplantation also resulted in improved survival and significant enzymatic delivery, by using both nontransduced murine NSCs [69] and human transduced NSCs expressing β-hexosaminidase [70]. Animals that received transduced human NSCs showed significant survival benefit, after both neonatal and adult transplantation. The improvement after transplantation of adult symptomatic Sandhoff mice [71] suggested its beneficial effect in symptomatic subjects with established disease, a more clinically appropriate situation than that modeled by neonatal transplant. Similarly, in Niemann-Pick type A disease, significant enzymatic levels were achieved with transplantation of transduced NSCs in acid sphingomyelinase-deficient mice [72].

Benefits of NSC transplantation were also seen in animal models of NCL, a family of neurodegenerative disorders that share the excessive accumulation of lipofuscin, with decreased accumulation of the toxic pigment upon transplantation of NCSs [73]. In that regards, a phase I clinical trial was initiated in 2006 for infantile and late infantile NCL, caused by deficiencies of the lysosomal enzymes palmitoyl-protein thioesterase 1 (PPT1) and tripeptidyl peptidase 1 (TTP1), respectively. The trial consisted of allograft of a banked human NSC line into the brains of six patients with advanced disease, who received 12 months of immunosuppression (NCT00337636). The study yielded a favorable safety outcome, with evidence of cell engraftment and survival up to 918 days after transplantation [16]. However, no functional benefit was seen in this phase 1 study, and the sponsor did not pursue a phase 2 efficacy assessment.

Krabbe disease is another lysosomal disorder that may comprise a feasible target for progenitor-based therapy. Krabbe's is a rapidly progressive disorder of the central white matter, caused by deficiency of galactocerebrosidase (GalC), which catalyzes the metabolism of galactocerebroside in oligodendrocytes. GalC deficiency results in severe demyelination as a consequence of oligodendrocyte death and is typically fatal within the first few years in affected children. In the twitcher mouse model of Krabbe disease, intracerebral transplantation of transduced murine neural stem progenitor cells with a β-GalC lentiviral vector resulted in detection of GalC levels in the brain and a modest improvement in survival. Although the cells migrated preferentially towards areas undergoing active demyelination, cell survival was poor at later time points, possibly due to the highly inflammatory milieu characteristic of

Fig. 15.2 (continued) saltatory conduction (Caspr, *red*; Kv1.2, *green*) (**e**) Electron micrograph of a 16-week-old homozygous shiverer mouse implanted perinatally with human OPCs. This image shows a resident shiverer axon with a densely compacted myelin sheath. (**f–i**) Myelination of shiverer mice by human iPSC-derived glial progenitor cells. (**f**) By 13 weeks of age, human iPSC-derived OPCs (hNA, *red*) matured into MBP-expressing oligodendroglia (*green*) throughout the subcortical white matter. (**g**) Confocal images of the callosal and capsular white matter of mice engrafted with hiPSC OPCs derived from line C27 [46] show dense donor-derived myelination. By 19 weeks of age, hiPSC oligodendroglia robustly myelinated axons (NF, *red*; MBP, *green*). (**h**) hiPSC-derived OPC myelination in a shiverer forebrain at 7 months. (**i**) Representative electron micrograph of a section through the pons of a 40-week-old shiverer mouse neonatally engrafted with C27 hiPSC OPCs, showing donor-derived compact myelin with evident major dense lines, ensheathing mouse axons. Scale: (**d**), 5 μm. (**e**), 1 μm; (**f**), 100 μm; (**g**), 50 μm; (**i**), 200 nm. Adapted from [64] (**a–b**); [19] (**c–d**); [29] (**e**); [46] (**f–i**)

Krabbe's [74]. A later study showed better results using genetically engineered murine and human NSCs, with evidence of cell survival, increased lifespan, and restoration of enzymatic levels [75].

Nonetheless, therapy solely directed towards CNS disease in Krabbe's has yielded only modest improvements in survival, likely due to progression of concurrent peripheral nerve disease. To address the latter issue, hematopoietic and umbilical cord stem cell transplants have been used as alternative sources for enzyme replacement, as these systemically administered cells will engraft both the CNS and PNS. The mechanism relies on the ability of peripheral monocyte-derived macrophages to populate the CNS and integrate as microglial cells, which can act as vehicles for enzyme delivery. That said, their rate of CNS colonization is slow, and their persistence largely perivascular, so patient responses to this strategy are highly variable. Nonetheless, allogeneic umbilical cord stem cell transplants have been shown to slow disease progression in infants with Krabbe disease, though these have been effective only when performed early in the disease course, before symptom onset [76]. Indeed, the lack of improvement seen in infants transplanted once already symptomatic emphasizes the need to initiate treatment early in the course of disease [77]. For later stages, delivering with cells capable of repairing extant damage while restoring wild-type enzyme will be needed. In such cases, GPCs may prove effective cellular vectors, at least for the CNS component of disease, given their ability to restore deficient enzyme levels while mediating remyelination of surviving axons. One might envision dual therapy with centrally administered GPCs and peripherally administered hematopoietic or umbilical stem cell transplants as having the greatest potential for long-term and durable clinical benefit.

Metachromatic leukodystrophy (MLD) is yet another potential target of neural stem and progenitor-based cell therapy. MLD is caused by deficiency of arylsulfatase A, which is necessary for breakdown of cerebroside 3-sulfate; its deficiency results in the accumulation of cerebroside in myelinating cells of both the CNS and PNS as well as, to a much a lesser extent, the visceral organs. In preclinical studies, intracerebral grafts of GPCs improved the clinical phenotype of MLD experimental models, with enzymatic rescue of arylsulfatase A and restored sulfatide clearance [78]. In patients, however, the results have been less clear. A small cohort of adult and late juvenile-onset MLD patients who received allogeneic hematopoietic stem cell transplantation exhibited slowed disease progression compared to untreated siblings [79]. As with Krabbe's disease, the lack of adequate response seen in patients with more severe, earlier onset forms of MLD may reflect the slow rate of cerebral repopulation and hence enzyme replacement by donor monocytes and their derived microglia. Accordingly, transplantation of cells transduced to overexpress arylsulfatase A was used in one study of early onset MLD children; this study demonstrated improved outcome in late infantile onset cases, though the long-term safety of lentivirally transduced donor phenotypes remains unproven [80]. To address this issue, a phase I/II clinical trial is recruiting presymptomatic or early symptomatic subjects with late infantile and juvenile forms of MLD (NCT01560182).

Non-lysosomal Leukodystrophies The most prevalent among non-lysosomal leukodystrophies is adrenoleukodystrophy, both neonatal and X-linked, which are

characterized by abnormalities in fatty acid metabolism that reflect peroxisomal dysfunction. While central adrenoleukodystophy might prove amenable to GPC-based cell therapy, adrenoleukodystrophy typically includes peripheral nerve and muscle disease that would be unaffected by a centrally targeted approach. As such, only those cases in which central disease strongly predominated would be appropriate therapeutic targets, and these may be difficult to predict at the early stages at which patients might most benefit from treatment.

In contrast to the widespread pathology and hence challenging therapeutic options in adrenoleukodystrophy, vanishing white matter disease (VWMD) is almost exclusively central in nature, affecting only the brain. VWMD is an autosomal recessive disease caused by mutations affecting any of the five subunits of the eukaryotic translation initiation factor (EIF2B2), resulting in rapid neurological deterioration triggered by head trauma, fever, or other event stress inducing event [81]. The disease predominantly affects the white matter, often leading to cystic white matter degeneration and frank cavitation. At a microscopic level, there is both oligodendrocytic [82] and astrocytic pathology [83]. As such, the implantation of GPCs may prove a viable approach towards replacing the genetically defective cells and restoring both normal glia and functional myelin to affected patients.

A variety of other non-lysosomal hereditary disorders of the white matter exist, which may be variably amenable to GPC-based cell therapy, depending upon the extent to which pathology is limited to glia, and whether the affected glial cells are replaceable by immigrating donor cells. While experimental models have supported the ability of healthy donor GPCs to replace either structurally- or enzymatically deficient host cells, the limits to this replacement capability are unclear. For instance, in leukodystrophies such as Alexander disease, in which GFAP-mutant defective astrocytes are insufficiently supportive of local oligodendrocytes, but are derived from GPCs that are otherwise normal until the point of astrocytic differentiation, will implanted wild-type donor GPCs be able to competitively dominate the host pool and effectively integrate? If not, then they will be unable to ever replace the host astrocytic pool and would hence be ineffective at rescuing the leukodystrophic white matter loss of Alexander's disease. On the other hand, if wild-type GPCs can in fact integrate within the Alexander's host, then this CNS-specific leukodystrophy might actually prove an optimal and appropriate target for cell therapy. Future studies will no doubt determine the breadth of disease targets potentially amenable to GPC-based cell replacement.

Autism Spectrum Disorders and Schizophrenia Children with autism spectrum disorders exhibit abnormalities in central myelin, as defined by both white matter volume and ultrastructure. Patients with autism can manifest reduced cortical white matter [84], enlargement of the corpus callosum [85], and widespread microstructural abnormalities, such as decreased fractional anisotropy and increased radial and mean diffusivity, as measured by diffusion tensor density [86]. Abnormal connectivity has been strongly suggested by many groups as the mechanistic basis underlying pathology in the brains of children with autism [84, 87], but the causative effect of such findings and the main cellular basis underlying these abnormalities are not completely understood. Similarly, in schizophrenia white matter abnormalities have

been reported [88, 89], with reduced fractional anisotropy reflecting microstructural disorganization [90, 91]. An important role of glia was also highlighted by differential expression of myelination-related genes in the postmortem prefrontal cortex of subjects with schizophrenia, suggesting that oligodendrocyte dysfunction may play a key role in schizophrenia [92, 93]. Although long considered a neuronal disorder, these findings suggest that abnormal glial cells may be the basis for, or at least significantly contribute to, the abnormalities in neural circuitry and function characteristic of schizophrenia. If so, then glial progenitor replacement therapy may evolve to have a role in the treatment of these neurodevelopmental disorders, by providing normal astrocytes and oligodendrocytes alike to brains in which abnormalities in each glial phenotype may contribute to circuit pathology. That said, it remains to be established whether glial grafts are capable of replacing resident glia in these disorders, and whether abnormal neuronal connectivity may be reversible and, if so, over what time window.

Acquired Developmental Disorders of Myelin Beyond genetic neurodevelopmental disorders, acquired disorders, which are both more frequent and prevalent than the hereditary and metabolic disorders, are important therapeutic candidates. Among those are inflammatory demyelinating disorders such as pediatric multiple sclerosis and acute disseminated encephalomyelitis, and the cerebral palsies, resulting from injury to the neonatal brain. The term cerebral palsy encompasses a group of partially overlapping disorders that include periventricular leukoencephalomalacia (PVL) and intraventricular hemorrhage due to germinal matrix hemorrhage (IVH) as well as hypoxic ischemic injury to the full-term brain. PVL is a significant health problem that causes a spectrum of motor disability, cognitive, and behavior symptoms in just under 10 % of children born at less than 32 weeks gestational age and/or with very low birth weights of <1500 g [94]. Macroscopically, PVL is characterized by diffuse astrogliotic disease and, to a lesser extent, microscopic necrosis and axonopathy [95]. Pre-myelinating NG2- and O4-expressing oligodendrocytes are the most abundant oligodendroglial lineage cells in the early third trimester brain, which is most at risk for developing PVL. These late-stage progenitors and immature oligodendrocytes appear to be especially particularly vulnerable to oxidative stress and excitotoxicity; in PVL they appear to undergo maturation arrest resulting in hypomyelination, particularly in the periventricular areas [96–98]. The number of oligodendroglia in these lesions is not decreased, but rather the cells appear to become arrested in an early stage of oligodendroglial maturation. Replacement of these abnormal cells by cells capable of myelination may prove an attractive therapeutic option. However, this possibility needs to be specifically tested in pathologically accurate disease models, since changes in the extracellular matrix may render the environment of these lesions nonpermissive for the maturation of donor GPCs [95], while co-existent subplate and GABAergic neuronal injury in these brains may not be reversible [98, 99].

The developmental dysmaturation associated with prenatal periventricular white matter disease poses a particular challenge to the development of therapies intended to restore normal neural circuitry [100]. Modeling premature and neonatal brain

injury in a manner predictive of human pathology has proven difficult [101], so that studies predictive of the clinical benefits of neural or GPC transplantation are lacking. Large animals such as the sheep and the baboon best simulate this complex pathology [102, 103]; however, those models are expensive and difficult to maintain and have proven reliable in only a few laboratories experienced in their use [104].

Whereas PVL is largely a primary disorder of glia, hypoxic ischemic injury; Hypoxic ischemic injury affecting term neonates is distinct from that of PVL, in that it involves grey matter to a larger extent, and hence affects a panoply of neuronal and glial phenotypes. The basal ganglia, thalami, and cerebral cortex tend to be predominantly affected, although there is also global white matter involvement [105]. Mixed glial and neuronal populations may thus be necessary for cell replacement, and as such the development of cell-based strategies for the treatment of hypoxic-ischemic encephalopathy is neither as straightforward nor imminent as that for PVL. That said, intracerebral transplantation of NSCs has been evaluated in rodents, with modest motor improvements noted in some studies ([106, 107] for review). Similarly, NSCs derived from human ES cells survived in a rodent model of neonatal hypoxic ischemia, but the modest motor improvements observed appeared related to an enhancement of endogenous brain repair, rather than to cell replacement per se [108].

Clinical Considerations in the Use of Cell Transplantation for Developmental Myelin Disorders

The lineage and phenotype, expansion potential, and developmental stage of transplanted cells all need to be matched to and appropriate for the disorder they are intended to treat. Though this would seem axiomatic, many preclinical studies attempt to use common cellular phenotypes, such as mesenchymal and NSCs, to treat a broad variety of pathophysiologically disparate disorders, often with inadequate consideration of which cells and stages might be best suited to provide functional and structural reconstitution for the disorder and pathology at hand.

In general, the sooner a diagnosis is made and the need for a transplant-based therapy is deemed appropriate, the earlier in the disease course at which a cellular intervention is applied, the more likely the ultimate therapeutic benefit. Yet as a practical matter, many of these disorders are not screened in the newborn period, and patients present only once symptomatic. As a result, while most preclinical studies of cell therapy for the leukodystrophies have focused on neonatal transplantation, relatively few have assessed therapeutic efficacy in subjects already manifesting disease. In addition, since many of the pediatric leukodystrophies in particular involve not only the nervous system but also both the peripheral nervous system and often visceral organs as well, combination therapies including parallel treatment strategies for each affected disease site need to be further developed.

Conclusion

In childhood disorders of myelin, oligodendroglial cells are either defective or diseased themselves, or impaired by virtue of paracrine associations with other impaired cells, most often diseased astrocytes upon which oligodendrocytes depend. In these cases, replacement with GPCs, capable of broad dispersal, stable integration, enzymatic replacement, and structural myelination, provides a promising therapeutic option. Indeed, the attractiveness of this strategy is heightened by the lack of therapeutic options otherwise available; most pediatric leukodystrophies are incurable and are associated with significant morbidity and early mortality. As such, those congenital disorders of myelin that have clear genetics and pathology may comprise favorable targets for therapy, since GPC transplants may permit the replacement of the mutant host cells by normal cells capable of functional myelination. That said, it is important to note that while experimental models have supported the colonization capability of implanted human donor cells, these studies have largely been done in the setting of xenografts. As a result, the extent to which allografted human donor cells will actually replace, rather than simply augment, host cell populations in disease settings remains unknown and will likely need to be defined on a disease-by-disease basis as cell therapeutics proceeds to the clinic.

Autologous transplants with the derivatives of genetically corrected iPSCs will provide further significant opportunities for treating allelic and monogenic disorders of myelin formation and maintenance. The primary hypomyelinating disorders of PMD and its variants, as well as the lysosomal and peroxisomal disorders of glial metabolism, should be particularly amenable to a strategy of transplanting autologous iPSC-derived GPCs after their genetic correction. On the other hand, polygenic disorders of less certain etiology and pathology, such as the autism spectrum disorders and schizophrenia, pose challenging targets for cell therapeutics, as their associated myelin abnormalities may be either cause or effect of the underlying disease process, and variably so across patients. Nonetheless, the increasing evidence of myelin pathology in these disorders suggests a potentially causal role for glial pathology and thus the possibility of a role for cell therapeutics. While the concurrent neuronal involvement and abnormal connectivity of the cognitive and neuropsychiatric disorders suggest the need for caution in pursuing a glial-focused strategy, the glial regulation of synaptic structure and strength is so intertwined with glial physiology, and with astrocytic function in particular, that we might readily envision the therapeutic potential of glial transplants in these disorders.

For acquired disorders of myelination, such as neonatal brain injury, those with predominant white matter involvement, periventricular leukomalacia in particular, are the most likely beneficiaries of glial cell therapy, whether of autologous or allogeneic cells. The most likely impediments to the use of glial progenitor transplant-based treatment strategy in the hypomyelinating cerebral palsies is not the cellular technology, but rather the twin difficulties of establishing and scaling up appropriate animal models and identifying appropriate patient populations. Predicting the course and prognosis of infants with PVL and germinal matrix loss

remains challenging, making clinical trial design more difficult than in the hereditary and metabolic disorders of myelin, whose relatively stereotypic natural histories may permit clearer determinations of therapeutic efficacy. It is this latter category of disease that is the most likely to comprise the first clinical targets for GPC-based therapeutics, which promises to significantly alter the course of these otherwise devastating diseases of childhood, offering the potential not only for relief, but potentially of frank rescue.

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Chapter 16
Neural Stem Cells for Spinal Cord Injury

Paul Lu, Ruhel Ahmad, and Mark H. Tuszynski

Abstract Neural stem cells (NSCs) are potentially attractive cell sources for reconstruction of injured spinal cord circuits. Recent studies demonstrate that NSCs can survive grafting into sites of severe spinal cord injury (SCI) and extend very large numbers of axons over substantial distances, forming synapses with host neurons below sites of injury. Reciprocally, host axons regenerate into the stem cell grafts and form synapses. New synaptic relays are thereby formed across the lesion site, improving functional outcomes even after severe SCI. Additional studies are in progress to establish long-term safety and to scale up grafting methods to the larger primate system. Accordingly, this work is on a translational path.

Keywords Neural stem cells • Spinal cord injury • Axonal growth • Synaptic connection • Neuronal relay

Introduction

Spinal cord injury (SCI) results in neuronal death and the transection or severe compression of axons that mediate motor, sensory, and autonomic function below the lesion. Since the pioneering work of Ramon y Cajal more than a century ago, it has

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been appreciated that severely injured axons of the adult central nervous system (CNS) fail to regenerate [1], resulting in permanent functional deficits below the level of injury. There are no approved clinical treatments for either acutely protecting neurons and axons after SCI, or for promoting their regeneration, constituting a target of great unmet medical need.

Recent progress in the field of stem cell research has opened new avenues in the development of potential treatments for SCI. Generally, neural stem cell (NSC) therapies offer three potential mechanisms for the treatment of SCI: (1) "reconstruction" of injured axonal pathways, inserting NSCs into lesion sites to act as neural relays across the injury site; (2) remyelination of spared host axons surrounding sites of injury, or (3) neuroprotection, if administered sufficiently early after injury. This chapter will primarily focus on the use of NSCs to form functional neural relays across sites of injury and the remarkable ability of grafts of early stage neurons to extend new axons into the host spinal cord. We will more briefly discuss remyelinating and neuroprotective approaches that have already begun clinical trials.

Isolation and Characteristics of Neural Stem Cells

NSCs are early stage cells of the nervous system that are capable of self-renewal and differentiation into both neurons and glia [2] (Fig. 16.1). NSCs arise from embryonic stem cells (ESCs), one of the earliest stages of organismal development. ESCs can be isolated from the inner cell mass cells of a developing blastocyst and have been cultured from human, nonhuman primate, rat, mouse, and other species [4]. Under the influence of specific sets of transcription factors, subsets of ESCs will differentiate into multipotent NSCs. NSCs exist in the developing nervous system for a time period of days to months, depending on the species, and expand rapidly in number to constitute and fill out the primordial nervous system [2]. After several cycles of cell division, NSCs enter the intermediate neural progenitor cell stage in which the capacity to undergo self-renewal becomes limited, and neural progenitor cells begin to commit to one of two specific lineages: neuronal restricted progenitor cells that generate exclusively neurons or glial restricted progenitor cells that generate exclusively astrocytes and oligodendrocytes [5, 6]. At this stage, cells can undergo only a limited number of additional divisions and then proceed to differentiate fully and adopt their mature phenotype.

Protocols exist today to drive cultured NSCs to specific mature phenotypes. For example, cells can be driven toward dopaminergic neurons as candidate cell replacement therapies in Parkinson's disease [7, 8], striatal interneurons in Huntington's disease [9], or cortical neurons in models of stroke [10]. Some of these strategies are moving toward clinical trials (see Chaps. 11–13 and 17). NSCs can also be driven toward specific glial fates, allowing oligodendrocyte replacement in congenital or adult dysmyelinating conditions of the nervous system (see Chaps. 14–15).

For spinal cord applications, two potential NSC replacement strategies have been tested experimentally: (1) grafts of multipotent NSCs, exploring the hypothesis that grafted cells will replace neurons, astrocytes, and oligodendroctyes at the site of

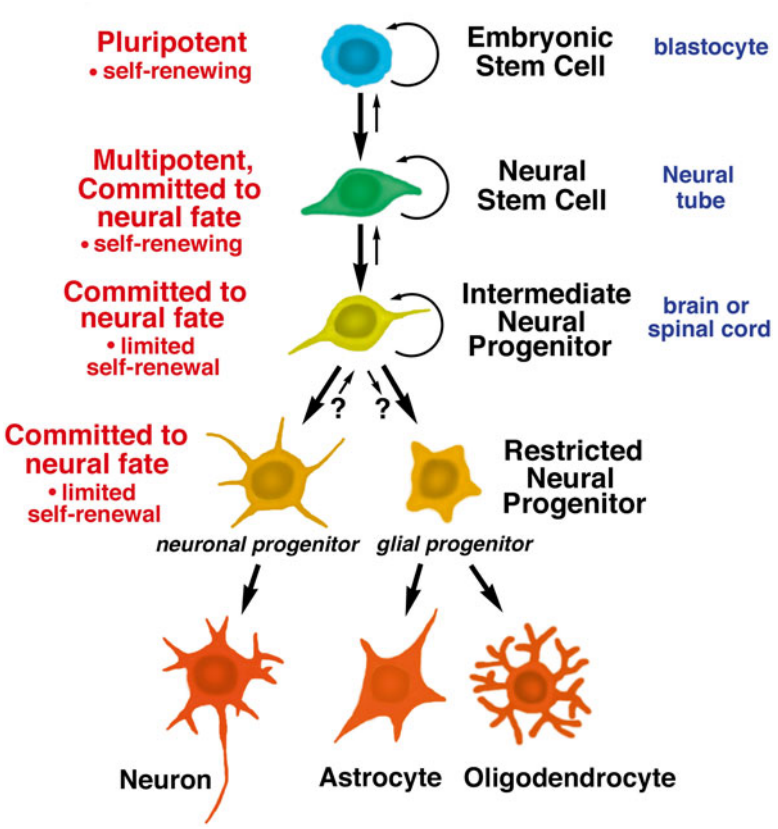

Fig. 16.1 Stages of development of embryonic and neural stem cells (modified from [3]). All stem cells are capable of division and self-renewal; daughter cells may differentiate into specific lineages, including neural progenitors, then neuronal cells and glial cells

injury and form new neural relays across the lesion site [11–13] or (2) grafts of cells driven toward oligodendrocyte progenitor cells (OPCs), exploring the hypothesis that grafted cells will remyelinate spared host axons traversing the injury region to partially restore function [14]. A third possibility is that NSCs or their derivatives might secrete neuroprotective substances such as growth factors [15, 16] that will provide neuroprotection, if grafted very early after SCI.

Most early work exploring the therapeutic potential of NSCs in models of SCI used cells originally derived from developing spinal cord or brain [17–19]. In the last several years, several alternative cell sources for generating NSCs or their derivatives have arisen (Table 16.1). These include: (1) NSCs directly derived from the ESCs [20–25]; (2) NSCs derived from induced pluripotent stem cells (iPSCs), which in turn are derived from mature a somatic cell (fibroblast, adipocyte, marrow stromal cell, etc.) using defined sets of transcription factors [22]; and (3) NSCs that are *directly differentiated* from a somatic cell using specific sets of transcription factors [26–30]. These are described below in more detail.

Table 16.1 Sources of neural stem cells and neural progenitor cells

Source	Cell types obtained
Direct isolation from the developing spinal cord and brain	Multipotent neural progenitor cells
Embryonic stem cells	Neural stem cells and their derivatives
Induced pluripotent stem cells	Neural stem cells and their derivatives
Directed differentiation (induced neural stem cells)	Neural stem cells and their derivatives

Neural Stem Cells Directly Isolated from the Developing Spinal Cord

Over developmental days 11–18 in rodents, a population of NSCs can be obtained that are transitioning to progressively more fate-committed neuronal restricted and glial restricted progenitor cells [5, 31]. An advantage of the developing spinal cord as a source material for use in SCI models is that the cells generally adopt spinal cord-specific cell fates, noted in the early SCI literature [17–19] and more recently by detailed transcription factor analysis [32–34]. This becomes increasingly important when attempting to form neural synaptic relays across lesion sites, because excitatory interneurons of the spinal cord may represent an ideal cell type for forming functional relays. Multipotent neural progenitor cells can be isolated from the developing human spinal cord at developmental stages of 8–10 weeks [35]. Indeed, such cells have now been used in human clinical trials of NSC therapy for amyotrophic lateral sclerosis (see Chap. 13).

Embryonic Stem Cells Driven to Neural Stem Cells

ESCs are derived from the inner cell mass of the early blastocyst and are able to proliferate for a long period of time and differentiate into almost all cell types, including NSCs. There are many lines of ESCs from human and mouse, but very few from rats, although rats serve as a very popular animal model for SCI. Early studies generated OPCs from ESCs for remyelination of spared axons after SCI [14, 36]. ESCs can also generate NSCs for both neuronal and glia replacement therapy for SCI [37].

Induced Pluripotent Stem Cells Driven to Neural Stem Cells

In 2006, Yamanaka and colleagues described the first successful generation of pluripotent stem cells from adult, fully differentiated fibroblasts using a set of four transcription factors [38], termed iPSCs. The initial report identified the ability to derive NSCs from iPSCs, raising the possibility of replacing the injured adult

nervous system with self-derived neurons and glia. Work remains to be done to generate spinal cord-specific fated NSCs from iPSCs, but when these tools are available, they will constitute a highly intriguing and perhaps optimal cell source for use in models of SCI.

Direct Differentiation of Somatic Cells into Neural Stem Cells

More recently, techniques have been developed to directly drive mature (even "post-mitotic") somatic cell types to other fates [39]. Fibroblasts, hematopoietic cells or other cell types can be directly converted to NSCs [26, 28] or even more mature neuronal fates [40] using specified sets of transcription factors. For example, fibroblasts obtained from skin biopsies can be established in culture and induced to express various combinations of transcription factors such as Sox2, Klf4, c-Myc, Brn2, Brn4, E47/Tcf3, and FoxG1. With exposure to optimized cell substrates, growth factors, and small molecules, one can eventually isolate NSCs after in vitro growth periods generally ranging from 1 to 2 months.

Thus, ESC-derived NSCs, iPSC-derived NSCs, induced NSCs, and spinal cord-derived multipotent neural progenitor cells all constitute potential cell sources for the delivery to the injured spinal cord. More information remains to be established regarding the unique properties of each type of NSCs, whether epigenetic factors influence the expression of maintenance of their mature fates, and their safety with regard to tumorigenicity and chromosomal stability.

Finally, protocols exist for deriving more mature cell fates starting from NSCs. For example, a number of studies in the SCI field have focused on remyelination of spared axons by implantation of oligodendrocyte precursor cells [14, 36]. Oligodendrocyte precursor cells are generally obtained by placing NSCs in culture conditions that direct their differentiation into oligodendrocyte lineages. Factors that drive NSCs to oligodendrocyte precursors include growth factors such as hepatocyte growth factor (HGF) [41] or ciliary neurotrophic factor (CNTF) and thyroid hormone [42, 43] and substrates consisting of laminin and poly-L-lysine [14] or laminin, collagen, and Nidogen-1 [44].

In Vivo Studies of Neural Stem Cell Therapy for Spinal Cord Injury

Formation of Novel Synaptic Relays Across Sites of Injury

Early SCI studies from the 1950s through the 1980s used "fetal" spinal cord transplants in an effort to restore neural conduction across sites of injury [18, 19, 45]. These studies generally used implants of solid pieces of spinal cord parenchyma removed from the E12–E16 spinal cord, although in some studies cells were

dissociated prior to implantation. This pioneering work resulted in some reports of functional improvement, although variable graft survival and the use of incomplete spinal cord lesion models often precluded more clear interpretations. Tools at the time to track the fate and extension of axonal processes from grafts were limited. Newly formed synapses between fetal spinal cord implants and host neurons could be appreciated at the ultrastructural level, although the frequency and impact of these connections were difficult to appreciate.

The development of modern tools of neuroscience permitted a reassessment of the hypothesis that early stage neural cells, grafted to the injured adult spinal cord, would enable formation of new synaptic relays across sites of injury to improve functional outcomes. To test this hypothesis, we utilized donor cells from transgenic rats expressing GFP in all cells under the ubiquitin promoter [13, 46, 47]. Donor cells came from the E14 spinal cord of GFP ubiquitin rats, which at this age consist of a mixture of NSCs and cells committed to either spinal cord neuronal fates [neuronal restricted precursors (NRPs)] [5] or glial fates [glial restricted precursors (GRPs)] [6]. Cells from the E14 spinal cord are capable of dividing several times to generate additional neural cells, but then stop dividing and do not continue to propagate a NSC population.

Spinal cords from E14 GFP transgenic rats were harvested, immediately dissociated, and implanted in vivo. The cells were grafted into a model of T3 spinal cord complete transection to most accurately model the severe, near-complete nature of most human injuries. Moreover, cells were grafted into injury sites 2 weeks after the initial lesion, to more accurately model the delayed clinical time points at which neurosurgical intervention might be optimal, after stabilization of initial trauma.

However, initial efforts to graft cells resulted in very little cell survival in the lesion site.

We worked to develop new methods to optimize cell engraftment and survival. Over several iterations, we developed a grafting "cocktail" consisting of 10 growth factors (Table 16.2) into which freshly dissociated cells were added. Further, we added fibrinogen and thrombin to the graft cell/cocktail mix, providing a rapidly gelling matrix that retained graft cells in the lesion cavity. Moreover, this approach more evenly distributed cells throughout the lesion site.

Table 16.2 Growth factor grafting cocktail

Category	Name	Concentration
Neurotrophins	BDNF	50 μg/ml
	NT-3	50 μg/ml
	GDNF	10 μg/ml
	IGF-1	10 μg/ml
Neural stem cell proliferation factors	bFGF	10 μg/ml
	EGF	10 μg/ml
Angiogenesis factors	PDGF	10 μg/ml
	aFGF	10 μg/ml
	HGF	10 μg/ml
Anti-apoptotic	Calpain inhibitor	50 μM

Using these methods, consistent engraftment of multipotent neural progenitor cells in the lesion site was achieved. When assessed 6–7 weeks later, an astonishing number of neural progenitor cell-derived axons emerged from the lesion site and extended both caudally and rostrally from the lesion site (Fig. 16.2) [13]. Axons grew through *lesioned* white matter caudal to the injury for distances of more than 27 mm (nine segments) in the caudal direction and 20 mm (seven spinal segments) in the rostral direction. 29,000 GFP-labeled axons emerged from the graft in the caudal direction, quantified at a distance 0.5 mm caudal to the lesion. Of the emerging axons, approximately 22 % of progenitor cell-derived axons became myelinated by host oligodendrocytes at a distance 3 mm caudal to the graft (unpublished data). In contrast, our preceding efforts to promote *host* axonal regeneration into and beyond lesion sites resulted in the growth of approximately 300 axons for distances of 1 mm beyond the lesion site [48]. Thus, the growth capacity of early stage neural

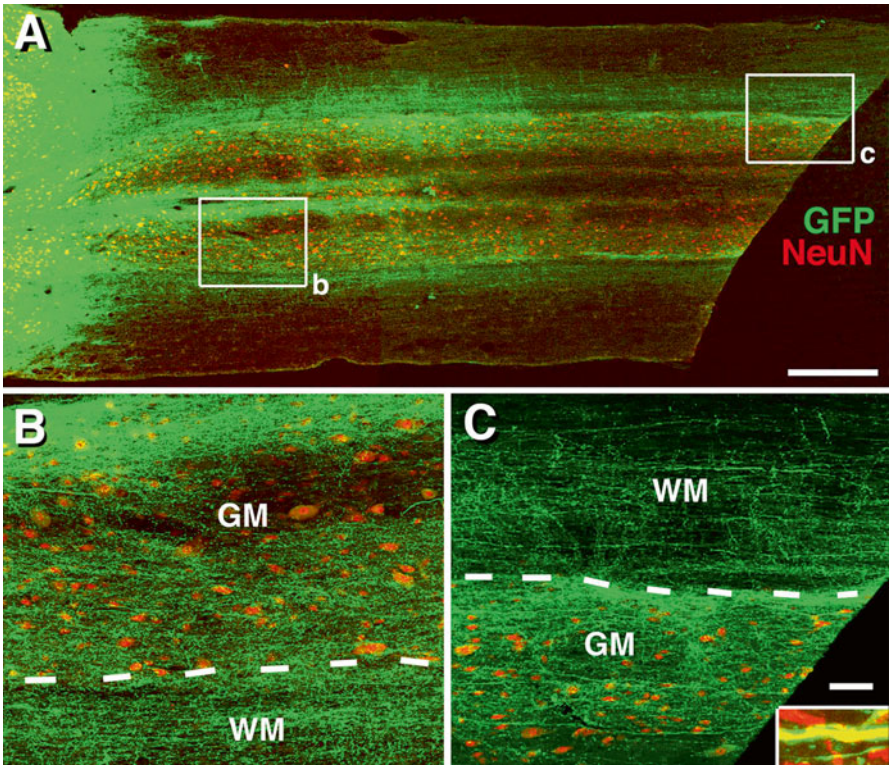

Fig. 16.2 Extensive long-distance axonal outgrowth from neural stem cell grafts. (**A**) *GFP* and *NeuN* immunolabeling reveals that *GFP*-expressing neural stem cell grafts robustly extend axons into the host spinal cord rostral and caudal to the T3 complete transection site (caudal shown) over the 12 mm length of the horizontal section. (**B**–**C**) Higher magnification views from *boxed area* in (**A**). Extensive regions of the host spinal cord contain graft-derived projections in white matter (WM) and gray matter (GM). Inset shows that *GFP*-labeled projections arising from grafts express neurofilament (*NF*), confirming their identity as axons. Scale bar = 550 μm (**A**), 60 μm (**B**–**C**)

progenitor cells exceeds that of adult regenerating axons, using these measures, by approximately 100-fold. Moreover, axons extended rapidly, growing at a rate of 1–2 mm per day [13].

Neural progenitor-derived axons formed synapses with host neurons located caudal to the injury, demonstrated both by the localization of synaptic markers and ultrastructural studies (Fig. 16.3). Thus, axons emerging from grafts of neural progenitor cells exhibit a truly unprecedented ability to extend large numbers of axons over very long distances through the injured nervous system.

Neural progenitor cells grafted into the lesion site generated both neurons and glia [13]. Approximately, 28 % of GFP-labeled neural progenitor cell grafts exhibited mature neuronal markers after 6 weeks, 27 % expressed mature oligodendrocyte markers, and 16 % expressed astrocyte markers (Fig. 16.4).

Reciprocally, host axons regenerated into NSC grafts, including reticulospinal axons that influence spinal motor control [13], and corticospinal axons that are the most important motor control system in humans (Fig. 16.5). The latter finding is of substantial significance, since it has been extraordinarily difficult to elicit regeneration of corticospinal axons after SCI [49–51].

Summarizing, host axons regenerate into grafts of multipotent neural progenitor cells placed in sites of complete spinal cord transection and form synapses with grafted cells. In turn, axons of multipotent neural progenitor cells extend out of the lesion site and form synapses with host neurons below the lesion. This could establish a new synaptic relay across the lesion site. To address this possibility, we stimulated the spinal cord at C7, four spinal cord segments above the lesion site, and measured responses at T6, three spinal segments below the lesion site. We detected responses below the lesion in three quarters of completely transected animals (Fig. 16.5). Confirming the origin of these responses from formation of new relays, the responses were entirely abolished by re-transecting the spinal cord *above* the graft

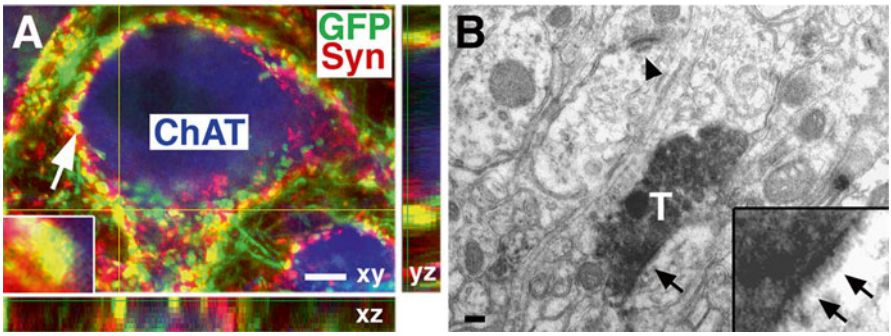

Fig. 16.3 Synapse formation of graft-derived axons with host neurons. (**A**) A *z*-stack image triple labeled for *GFP*, synaptophysin (*Syn*, inset), and *ChAT*, indicating co-association of graft-derived axons with a synaptic marker in direct association with host motor neurons (*arrowhead* indicates one of several examples). (**B**) Electron microscopy confirms that DAB-labeled *GFP*-expressing axon terminals form synapses (*arrows*) with host dendrites. *Arrowhead* indicates a separate, host–host synapse. Scale bar: (**A**), 8 μm; (**B**), 200 nm

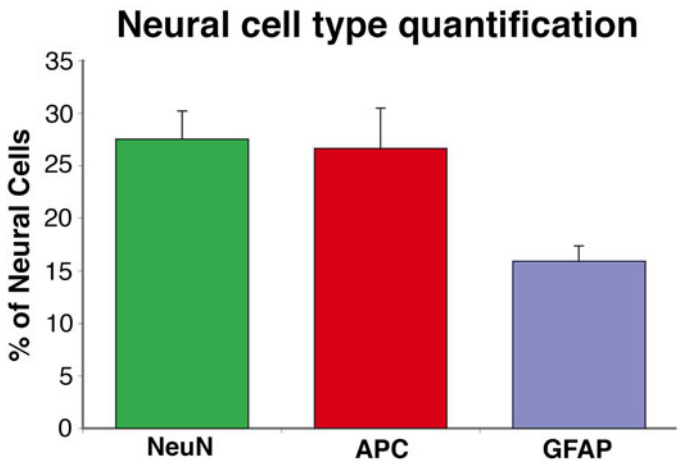

Fig. 16.4 Neural cell differentiation. Neuronal and glial phenotype quantification. Approximately, 28 % of grafted cells express the neuron-specific marker NeuN when assessed 6 weeks post-grafting, while 27 % of cells express the mature oligodendroglial marker APC and 16 % express the astrocyte marker GFAP

(Fig. 16.5). The ability to restore conduction across the lesion site also resulted in a measurable functional benefit: on the 21-point BBB locomotor scale [13]: grafted animals exhibited a significant 5.5 improvement in function compared to lesioned controls (Fig. 16.5). Once again, confirming that new neural relays across the lesion generated this functional recovery, re-transection slightly above the neural progenitor cell implant abolished the functional effect (Fig. 16.5).

These findings strongly support the concept that grafts of NSCs or progenitor cells can support the formation of novel relays across sites of even the most severe form of SCI, complete transection.

Grafts of Human ESC- or iPSC-Derived NSCs to Sites of SCI

The preceding findings indicate that early stage rodent neurons exhibit a robust intrinsic capacity to extend large numbers of axons over very long distances through degenerating white matter of the injured spinal cord caudal to a severe injury site. These findings have clear translational potential. To more fully understand their translational potential, we sought to determine whether NSCs generated from other source cells, including ESC lines or iPSCs, also exhibit similar growth properties when grafted to sites of SCI.

We grafted NSCs derived from the "approved" human HUES7 ESC line that was isolated at the Harvard University [52] to rats with complete spinal cord hemisections at the C5 spinal cord level [13]. When grafted in a fibrinogen/thrombin matrix containing a growth factor cocktail, HUES7 cells, like rat spinal cord-derived mul-

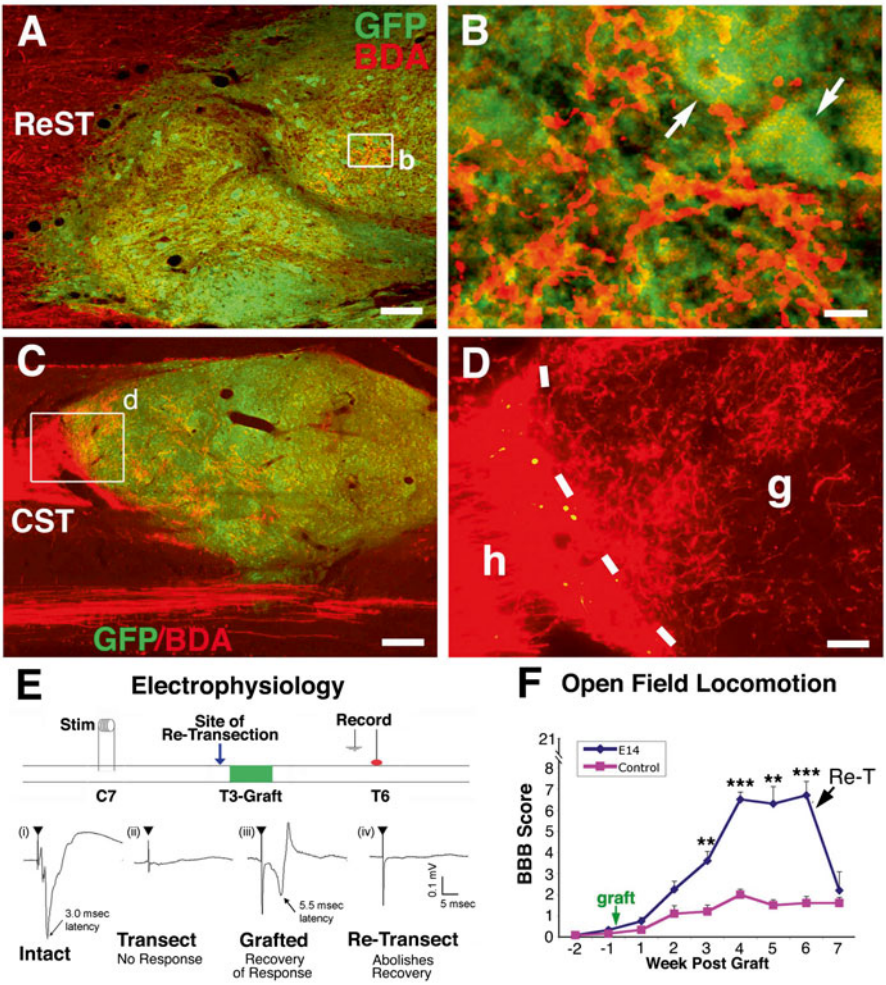

Fig. 16.5 Host axonal regeneration, electrophysiology, and behavior. (**A**–**B**) Host reticulospinal tract (ReST) axons labeled with *BDA* regenerate into *GFP*-expressing neural stem cell grafts in site of T3 complete transection (**B**, from *boxed area* of panel **A**; *arrows* indicate *GFP*-labeled grafted cells with neuronal morphology). (**C**–**D**) Host corticospinal tract (CST) axons labeled with *BDA* robustly regenerate into *GFP*-expressing neural stem cell grafts in site of C3 dorsal column lesion **D** is from *boxed area* of panel **C**. *Dashed lines* in panel **D** indicate host (**h**) and graft (**g**) interface. Scale bar: **A**, 64 μm; **B**, 12 μm; **C**, 300 μm; **D**, 62 μm. (**E**) Electrophysiological transmission across the T3 complete lesion site: (i) In intact animals, stimulation at C7 evoked a short latency (~3.0 ms), large amplitude response at T6. (ii) Transection of the cord at T3 completely abolished this response. (iii) In four of six lesion/grafted animals, recovery of an evoked response of prolonged latency (~5.5 ms) was observed. (iv) Re-transection of the spinal cord at T3, just rostral to the graft (*green arrow*), abolished the recovered evoked response. (**F**) BBB scores of hindlimb after T3 complete transection show significant improvement in subjects that received neural stem cell grafts (E14, $n=6$) compared to lesioned controls ($n=6$). Re-transection (*arrow*, Re-T) at rostral interface of graft with host abolishes functional improvements when assessed 1 week later ($^{**}p<0.01$, $^{***}p<0.001$)

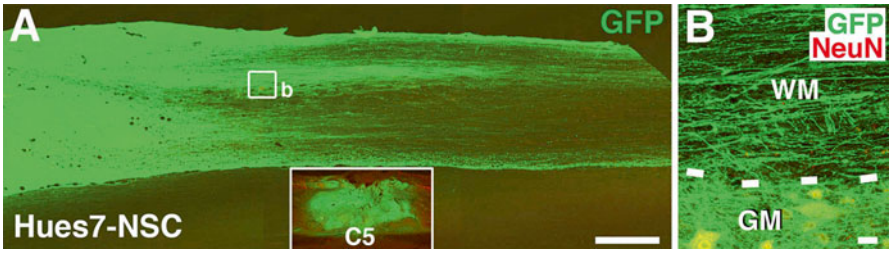

Fig. 16.6 Axonal growth of human ESC-derived neural stem cells into rat injured spinal cord. (**A**) *GFP*-labeled human embryonic stem cells (ESCs) (HUES7) grafted into sites of C5 hemisection spinal cord injury (*inset*) extend large numbers of projections into the host spinal cord. (**B**) A higher magnification view from the *boxed area* in panel (**A**) showing growth of human axons in host white matter (WM) and gray matter (GM, labeled with neuronal marker NeuN). Scale bar: (**A**), 750 μm; (**B**), 20 μm

tipotent neural progenitor cells, extended extraordinarily large numbers of axons over very long distances in the rat spinal cord (Fig. 16.6).

We next generated human NSCs from cultures of human iPSCs [53]. Human iPSCs were generated from fibroblasts obtained from a skin biopsy of a healthy, 86 year-old male. These cells were cultured on PA6 cells, and NSCs were purified using fluorescent activated cell sorting for the NSC markers $CD184^{+}$, $CD15^{+}$, $CD44^{-}$, and $CD27^{-}$. When grafted to sites of C5 complete hemisection lesions, these iPSC-derived NSCs exhibited the most extensive growth properties yet identified and extended axons out from the lesion site over the entire extent of the rat neuraxis (Fig. 16.7).

Thus, NSCs from various species, including mice, rat, and humans, exhibit an ability to survive engraftment to sites of severe SCI, and extend axons in large numbers and over very long distances. Host axons also regenerate into the stem cell grafts in the lesion site, forming new synapses. These approaches have substantial value to potentially serve as a means of forming novel neural relays across sites of SCI. Two separate lines of additional research are suggested by these findings: first, we aim to understand *mechanisms* underlying the ability of NSCs to extend axons so extensively through the injured adult CNS. A greater understanding of these mechanisms could identify novel avenues to further augment regeneration of injured adult axons. Second, we aim to perform additional efficacy, safety, and toxicity studies to determine whether these approaches merit testing in human clinical trials. These two avenues of research are addressed in the next section.

Mechanistic Studies of Neural Stem Cell-Induced Axonal Growth

Why do early stage neurons extend axons to a remarkable extent through the injured adult spinal cord, whereas host axons fail to regenerate? An understanding of mechanisms underlying stem cell-derived axon growth could be of great value in

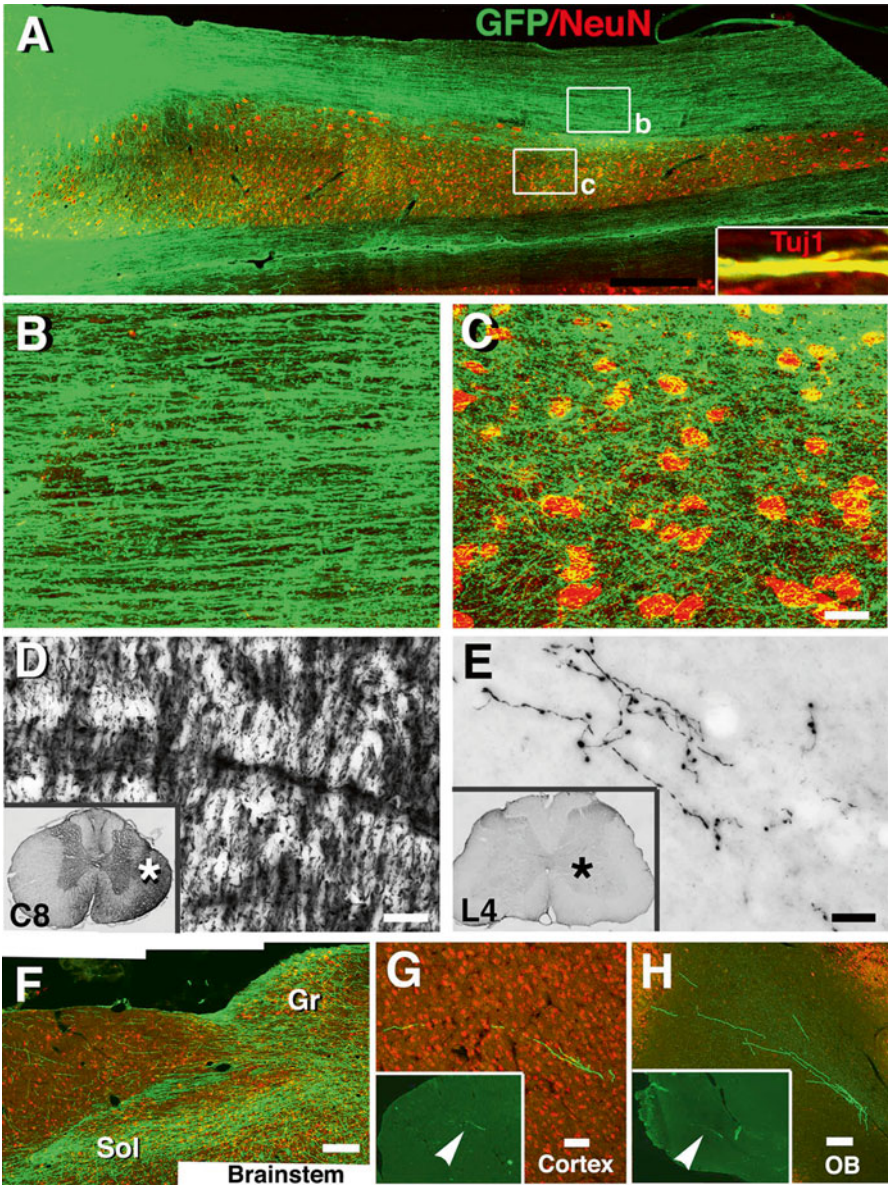

Fig. 16.7 Long-distance axonal growth of human IPSC-derived neural stem cells in sites of spinal cord injury. (**A**–**C**) Very large numbers of *GFP*-labeled axons extend caudally into the host spinal cord (**B**) white matter and (**C**) gray matter (region of *NeuN* labeling). Insets in panel (**A**) indicate that axons co-localize with *Tuj1*. (**D**–**E**) Light-level *GFP* immunolabeling of human iPSC-derived axons in coronal sections shows very large numbers of axons extending into *caudal* host spinal cord. Insets in each panel show the sampled region from which higher magnification views were obtained: (**D**) C8 and (**E**) L4. (**F**–**H**) Fluorescent *GFP* labeled human iPSC-derived axons extend *rostrally* into brain in sagittal sections at (**F**) the brainstem (*Gr* gracile nuclei, *Sol* solitary nuclei). (**G**) cortex, and (**H**) olfactory bulb (OB). (**C**), 600 μm; (**D**–**E**), 32 μm; (**F**), 250 μm; (**G**), 20 μm. Scale bar: (**A**), 600 μm; (**B**–**C**), 32 μm; (**D**), 20 μm; (**E**), 60 μm; (**F**), 120 μm; (**G**–**H**), 100 μm

advancing regenerative therapies. We and others are engaged in RNA sequencing studies of NSCs to illuminate genome-wide patterns of transcription that are associated with an active growth state. The key regulatory mechanisms that generate chromatin configurations representing an early, growth-permissive state could lead to novel therapies to reprogram injured adult neurons to regenerate. Data from these experimental approaches are just beginning to become available.

Similarly, why does the presence of the NSC graft enable regeneration of host axonal populations including corticospinal axons, which previously have been unresponsive to efforts to elicit their regeneration? A systematic study of cell–cell interactions, extracellular matrix components, and receptor-ligand binding of regenerating axons with cells in the graft can provide new insight into basic molecular mechanisms that have, for the first time, enabled regeneration of refractory host axons into NSC grafts.

The Path of Neural Stem Cell Translation to the Clinic

Additional studies will enable the optimal design of clinical translational approaches for the treatment of SCI.

First, replication of functional recovery by independent observers will enhance confidence that the proposed approaches merit clinical translation.

Second, the SCI regeneration field is, in a sense, provided an embarrassment of riches in the sheer number and distances of axons regeneration from implants of NSCs in sites of SCI. Might some of these extending axons result in adverse functional outcomes? It is important to generate high quality data regarding anatomical and functional outcomes in sensory systems, particularly nociceptive axons, after NSC grafting. How are pain outcomes influenced by NSCs? Many patients who sustain severe SCI exhibit chronic pain [54]; others do not. Data regarding outcomes of nociceptive measures will be important, as NSC grafts may improve, not alter, or even worsen outcomes. These are important functional endpoints to study. It is also important to examine effects of NSC grafts on autonomic outcomes, including bowel [55], bladder and sexual function [56], and spinal dysreflexia [57].

Another important question to address is the time frame over which grafts of human NSCs mature after grafting to sites of SCI. Whereas rodent NSCs express mature neuronal and glial markers at time points of only 1 month after in vivo grafting, a typical time frame for rodent stem cell maturation, human NSCs mature over extended time periods of months, even years [58]. At what rate will human NSCs mature when grafted to sites of SCI? If maturation occurs over years, clinical trial design will need to take this consideration into account.

Long-term safety and toxicity of NSC grafting approaches also require further study. Cell cultivation methods are needed that are clinically compatible and consistent, and that eliminate cells that retain ESC characteristics because the later could form teratomas in vivo [59–61]. Moreover, the best embryonic cell lines from which to generate NSCs should be identified, as different sources of ESCs exhibit distinct properties of controlled growth and chromosomal stability in vivo [62].

Another practical translational issue is that we obtained the best results when grafting NSCs in a fibrinogen/thrombin matrix containing a cocktail of 10 growth factor proteins. But for clinical translation, all 10 of these components would require standardized, GMP manufacturing and testing. A far simpler solution would involve the use of as few components of the cocktail as possible, optimally just one or two factors. Studies are in progress to identify the extent to which a reduction in the number of growth factors can still support graft survival in larger lesion sites.

Finally, scaling up of candidate cellular therapies to larger animal models could be essential in identifying parameters enabling stem cell grafting to the injured human spinal cord. Indeed, in preliminary studies we have found that methods developed for NSC engraftment to sites of rat SCI were inadequate in nonhuman primate models, and several procedural modifications have been required in the primate model to elicit good graft survival and fill of the lesion site. This work continues and will constitute an important component of the translational program moving forward.

In summary, NSC grafting to sites of SCI represents an unprecedented opportunity and challenge in promoting neural repair. Ongoing studies will determine the timing and development of optimized techniques for human translation.

Other Neural Stem Cell Approaches for SCI

A number of studies have reported other approaches to stem cell grafting for SCI. Most of these reports focus on remyelination of spared axons after SCI [14, 16, 63–65]. The vast majority of human SCI cases are severe in extent [66] and result in permanent functional loss below the level of the injury [67]. Anatomically, however, there are often spared strands of white matter along the outer edge of the spinal cord. Whether these strands of white matter contain demyelinated axons that could be recruited back to a functional state remains controversial [68]. The fact that intensive rehabilitation in some chronically injured patients can result in some improvement in function, albeit limited, supports the concept of rehabilitation or remyelination as potential mechanisms to improve outcomes in SCI patients.

Most NSC experiments performed by other groups have targeted NSC or OPC grafting into host spinal cord surrounding the lesion site, rather than grafting NSCs into the lesion site itself [12, 14, 65]. This is in contrast to our experimental approach, which aims to directly fill the lesion site and form new neural relays across the lesion. In general, several reports adopting peri-lesion grafting in rats have reported modest improvements in the BBB locomotor scale [12, 14, 65]. Three clinical trials have been initiated adopting these approaches (*clinicaltrials.gov*). One of these programs, initiated by Geron, Inc., was discontinued after treating only four patients. Another program completed Phase 1 safety trials in Switzerland and is proceeding to multicenter trials. A third program, begun recently in the United States, is treating patients at least 1 year after SCI, although there are no preclinical data to support this delayed time point for grafting. Results have not yet been reported. None of these trials is using growth factors or other measures to enhance stem cell engraftment or survival.

Future Perspectives

Extensive axonal growth from NSCs and the potential formation of new functional synaptic relays across lesion sites offer new hope for the treatment of SCI. Numerous opportunities and challenges are revealed by these findings. How will emerging axons be directed toward appropriate targets, and will their synapses be stabilized over time? Grafted NSCs in bioengineered matrices may be one solution for guiding appropriate projections across lesion sites [69, 70]. The enrichment of excitatory neuronal phenotypes in grafts may enhance the efficacy of functional relay formation, an endeavor that is currently beginning. Inappropriate contacts across lesion sites may also form, resulting in adverse consequences; these adverse effects have not been detected in animal studies to date, and it is possible that nonfunctional synapses are naturally eliminated. Rehabilitation may act to shape newly generated circuits to enhance functional outcomes, since training and activity influence new circuit formation during development. Enhancing the regeneration of adult host axons into NSC grafts may enhance the efficiency of neural relay circuits, and a greater understanding of stem cell-mediated mechanisms enabling host axonal regeneration could identify means of amplifying the extent of host axonal regeneration. We are in a new era in which extensive axonal outgrowth from spinal cord lesion sites is possible; directing and optimizing that growth is now the emerging challenge.

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Chapter 17
Marrow-Derived Mesenchymal Stromal Cells in the Treatment of Stroke

Steven C. Cramer

Abstract Mesenchymal stromal cells (MSC) are a multipotent stem cell that can be derived from several different tissues, including bone marrow. In preclinical studies, MSC have been found to improve outcome after stroke with a time window measured in days–weeks, in a manner robust across species, delivery route, allogenicity, and dose. These effects are achieved via multiple mechanisms in parallel, including paracrine elaboration of neurotrophins and remote immunomodulation. Human studies support the safety of MSC administration after stroke. Future trials can be designed with attention to key issues reviewed herein, including choices during MSC manufacture, and principles of brain repair.

Keywords Stroke • Recovery • Mesenchymal stromal cells • Stem cells • Review • Clinical trial • Neural repair

Stroke Is a Major Cause of Human Disability

Stroke is a major source of human morbidity and disability [1]. Intravenous (IV) tissue plasminogen activator (tPA) is approved for treatment of acute ischemic stroke in the USA, but as a result of factors such as the narrow therapeutic time window of 3–4.5 h, many patients do not access medical care in time to benefit from current acute stroke reperfusion therapies [2–4]. Furthermore, many patients receiving IV tPA nonetheless show long-term disability. Consequently, while efforts continue to increase the impact of acute stroke therapies, other studies are examining the potential utility of other classes of stroke therapies that have a wider therapeutic time window than IV tPA.

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Restorative therapies aim to improve patient outcomes by promoting the neural processes underlying behavioral recovery [5] rather than by modifying the extent of injury. Cell-based therapies are among the most studied categories of restorative therapy [6], and among these considerable attention has been drawn to mesenchymal stromal cells (MSC; also termed mesenchymal stem cells or multipotent stromal cells), which are adult non-hematopoietic pluripotent cells. Human MSC have been defined by the International Society for Cellular Therapy [7] on the basis of three criteria, specifying that the cells must:

1. Adhere to plastic in standard culture conditions;
2. Express surface antigens such as CD105, CD90, and CD 73 but not CD34, CD45, or HLA-DR; and
3. Be able to differentiate in vitro to osteoblasts, adipocytes, and chondroblasts.

Substantial research has been published examining MSC effects, in cerebrovascular disease and in other settings such as graft versus host disease, where MSC are the basis for the first clinically approved human stem cell therapy in North America [8]. The vast majority of this effort has focused on MSC as a restorative therapy.

MSC Have Multiple Mechanisms of Action

A substantial body of preclinical evidence suggests that MSC improve behavioral outcomes after experimental stroke [9] (Fig. 17.1A). This occurs via several different mechanisms in parallel, a potential advantage over pharmacological therapies that act via a single treatment mechanism [13–15]. One set of mechanisms pertains to local changes in the brain, including neurogenesis, synaptogenesis, and angiogenesis [16]. MSC orchestrate numerous cellular proliferative events such as subventricular and subgranular zone cell proliferation, consistent with endogenous neuronal precursors mobilization (Fig. 17.1B), and in addition reduce apoptosis [17–19]. MSC have been found to infiltrate ischemic brain regions and upregulate genes related to restorative events [20], resulting in increased local levels of numerous growth factors and chemokines. These paracrine events promote improved recovery after stroke, and when MSC are administered early post-stroke might also be associated with a neuroprotective effect and thus decreased infarct volume (Fig. 17.1C). MSC secrete extracellular matrix components that promote neuronal survival [21, 22]. Some preclinical studies suggest that these MSC mechanisms of action can be favorably enhanced through modifications such as gene insertion, addition of bioscaffolding, or by adding specific growth factors to the culture medium [23, 24]. However, although MSC can differentiate into several mesodermal lineages, these cells do not replace functional neurons or glia [25–27] despite showing surface markers and phenotypic characteristics of such cells [15, 17, 28–36].

A second set of mechanisms relates to immunomodulatory mechanisms, which given the distribution of MSC after systemic administration (see below) is often remote in nature [14, 37]. Numerous inflammatory events occur after stroke, both

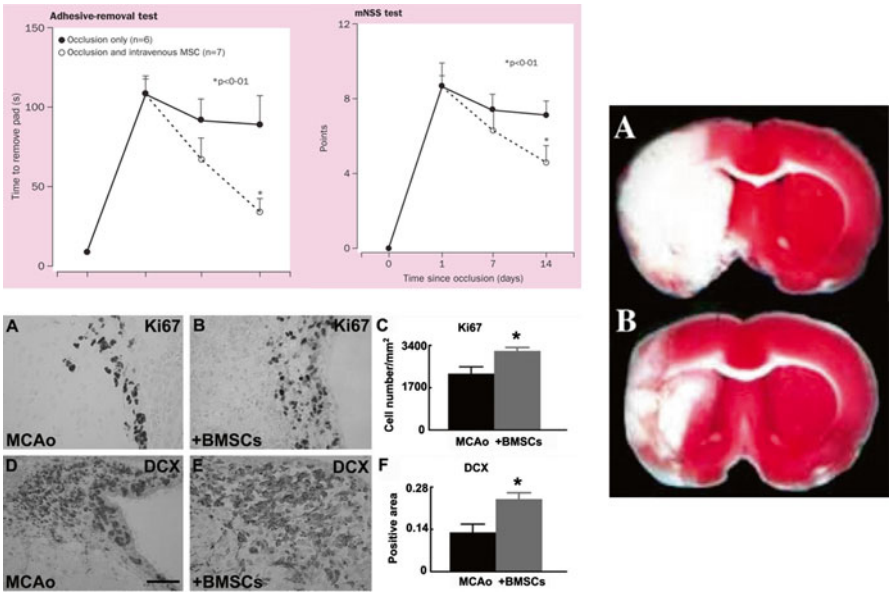

Fig. 17.1 (**A**, *Upper Left*)—Behavioral recovery after stroke is improved when rats are given MSC 24 h after onset of ischemic stroke. Error bars = SD. Reprinted with permission from Chopp and Li [10]. (**B**, *Lower Left*)—Cell proliferation and neurogenesis 8 days after stroke are increased when MSC are introduced 24 h after stroke. MSC significantly increased the number of proliferating Ki-67 positive cells in the subventricular zone (**A**–**C**) as well as the number of DCX-positive neuroblasts (**D**–**F**); $^{*}p < 0.05$ for MSC vs. control; scale bar (**A**, **B**, **D**, **E**) = 50 μm. Reprinted with permission from Shen et al. [11]. (**C**, *Right*)—Infarct volume is reduced when MSC are given early (6 h), but not late, after experimental ischemic stroke in rats. An example of early MSC administration is shown. Brain slices have been stained with 2,3,5-triphenyl tetrazolium chloride (TTC) to visualize the ischemic lesion 14 days post-stroke. The average infarct volume among rats receiving MSC 6 h post-stroke was 24 % smaller than values in control animals ($p < 0.01$). Reprinted with permission from Omori et al. [12]

within the brain and systemically [38–40]. MSC affect numerous immune events, for example, T-cell regulation via inhibiting T-cell proliferation, promoting T-cell regulatory effects, and exerting suppressive effects on CD4+ and CD8+ T-cells [41–44]. In addition, cytokines promote MSC immunoregulatory functions and lead to further T-cells and natural killer cell suppression [45]. MSC may also have effects on leukocyte proliferation [46–48] and B-cell and functions [49–52]. Other immunological effects include a shift in macrophage profiles [51, 53] as well as remote immunomodulation, e.g., from the lung [14, 37, 54] or spleen [55–58].

A key feature of MSC is that the cells themselves are generally considered to be relatively [51, 59, 60], though in some cases incompletely [61], immunoprivileged due to their expression of very low levels of HLA molecules [62, 63]. This opens the door to administration of allogeneic MSC, which greatly increases the translational potential for MSC, for example, by removing the need for immunosuppression; use

of allogeneic cells also allows for MSC transfusion on demand by eliminating lengthy delays required to expand autologous cells in vitro for individual patients, a major concern among patients with recent stroke, and so on.

Issues Related to MSC Generation and Transport

For biological therapies such as MSC, differences in manufacturing and handling can impact in vivo activity. Issues that have been identified as important include choice of culture media, duration of culture, storage conditions, transportation, and thawing conditions [64–67]. Furthermore, the number of cell divisions affects MSC in several ways including potency and viability [68, 69]. Culturing MSC in conditions that include animal products such as fetal bovine or calf serum is common and could theoretically introduce infectious or allergic risks; alternative approaches are under study [70, 71]. Factors specific to the donor of the bone marrow from which MSC are cultured can also affect the final cellular product [72, 73].

Preclinical Data

Distribution and Fate of Transfused MSC MSC can be administered intracerebrally (IC), intra-arterially (IA), or intravenously (IV). The fate and distribution pattern of MSC varies according to the route by which they are introduced [57, 74].

MSC are preferentially attracted to regions of brain ischemia, in part due to the general MSC characteristic of being attracted to sites of inflammation [75–77]. This is supported in part by expression by MSC of receptors [78] for inflammatory cytokines that are released after injury [79, 80]. Chemoattractants released by microglia and astrocytes in an infarct zone and its penumbra recruit MSC through receptors such as CXCR4 [81, 82]. Reduced integrity of the cerebral vasculature following stroke also contributes to the preferential MSC distribution to regions of brain ischemia, for example, via passive entrapment [37], although precise details by which MSC cross the blood–brain barrier require further study.

MSC are relatively large cells, having a diameter of 10–30 μm [83]. Not surprising, therefore, most MSC are passively entrapped in the lung vasculature following IV transfusion [56]. In healthy animals, IV MSC are mainly found in lungs and liver at 3 h postinjection; in lungs, liver, and spleen at 24 h; in bone marrow, spleen, and lung over the next several days; and in bone marrow, spleen, lung, bone, muscle, cartilage, and liver months thereafter [56, 84, 85]. After stroke, MSC also home to regions of brain injury [14, 76], with the vast majority found in the ischemic core and its peri-infarct boundary zone. MSC are predominantly eliminated by the kidneys [56]. One year after stroke, any surviving MSC are

generally found within the region of prior brain ischemia [86]. Introduction of MSC via the IA or the IC route is more invasive but does avoid first-pass pulmonary effects and so reduced the amount of MSC in lungs [87, 88].

Safety and Efficacy of MSC After Experimental Stroke A recent meta-analysis examined preclinical studies in which MSC was given after cerebral ischemia [89]. Data were extracted from studies identified on PubMed and ISI Web of Science. A Quality Score regarding study methodology was determined using the scale of Lees et al. [90], which defined 10 criteria based on STAIR guidelines [91, 92]: (1) publication in a peer-reviewed journal, (2) statements describing control of temperature, (3) random assignment of animals to treatment group, (4) allocation concealment, (5) blinded outcome assessment, (6) avoidance of anesthetics with known marked intrinsic neuroprotective properties, (7) use of animals with relevant comorbidities, (8) inclusion of a sample size calculation, (9) statement of compliance with animal welfare regulations, and (10) inclusion of a statement declaring presence or absence of any conflicts of interest. One point was given for each criterion reported; potential scores thus ranged from 0 to 10, with higher scores indicating greater methodological rigor. Effect size of MSC therapy was determined for four endpoints that appeared most often: (1) the modified Neurological Severity Score (mNSS), (2) Adhesive Removal test, (3) Rotarod test, and (4) infarct volume. For each, effect size was defined as the improvement in outcome in MSC-treated animals relative to untreated ischemic controls.

A total of 46 studies with 62 MSC treatment arms were identified. MSC improved outcomes in 44 of the 46 studies, and in 54 of the 62 treatment arms. Quality Score across these 46 studies had a median value of 5.5 and was not significantly related to route of MSC introduction, species receiving MSC, species of MSC source, time post-stroke, MSC immunogenicity (autologous vs. allogeneic), or MSC dose.

The mean effect size for MSC administration was consistently very large, with mean value of 1.78 for the modified Neurological Severity Score across 28 studies, 1.73 for the Adhesive Removal test across 22 studies, 1.02 for the Rotarod test across 14 studies, and 0.93 for infarct volume reduction across 43 studies (Fig. 17.2). Results were overall similar when analyses were restricted to studies that initiated MSC $\geq$ 24 h after stroke onset (Fig. 17.3). The effect size for the modified Neurological Severity Score was found to vary according to route of administration, although results remained significant for all three routes (Fig. 17.2a), and to vary inversely with MSC dose (Fig. 17.3b). In addition, Quality Score correlated with effect size for mNSS (Fig. 17.3a), indicating that the higher the study quality, the greater the improvement in behavioral recovery associated with MSC treatment. For infarct volume reduction (Fig. 17.2d), effects were highest when MSC were initiated in the early hours post-stroke, and remained significant 1 week post-stroke, but not with any later times of MSC initiation.

Funnel plots suggested significant ($p \leq 0.0001$) publication bias [93], with studies having a smaller effect size than current mean values being underreported. However, after adjusting for these asymmetries, mean effect sizes nonetheless remained very large (1.41 for the modified Neurological Severity Score, 1.23 for the Adhesive Removal test, 1.14 for the Rotarod test, and 0.62 for infarct volume reduction).

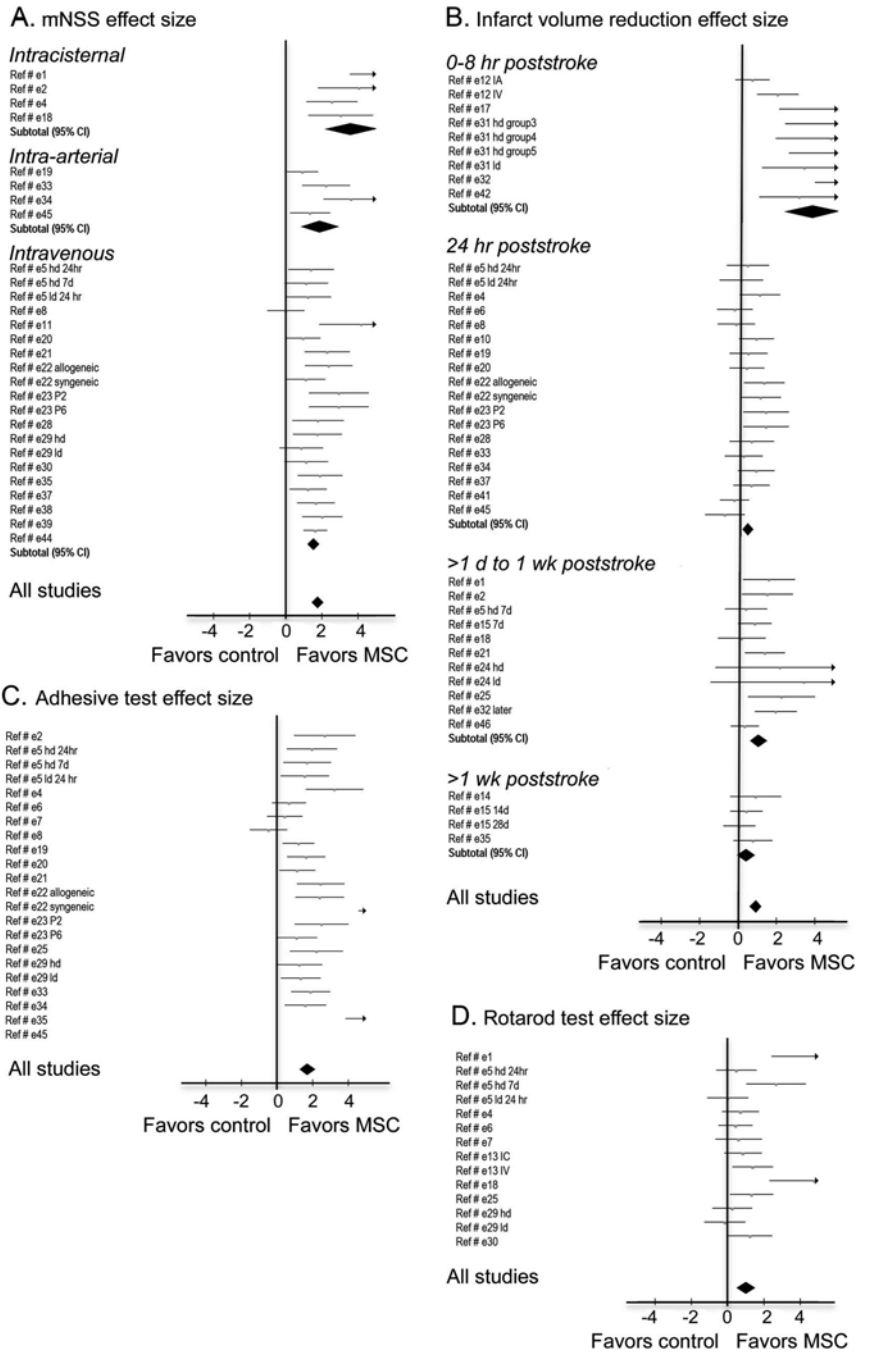

Fig. 17.2 Forest plots show mean effect size and 95 % CI for (**a**) modified Neurological Severity Scale, (**b**) Adhesive Removal test, (**c**) Rotarod test, and (**d**) infarct volume reduction. Values for effect size were very large and highly significant, and were robust across numerous variables such as (**a**) route of MSC administration and (**d**) time of MSC administration after stroke. *hd* higher dose group, *ld* lower dose group, *P2* two passages in culture, *P6* six passages in culture. From Vu et al. [89], with permission

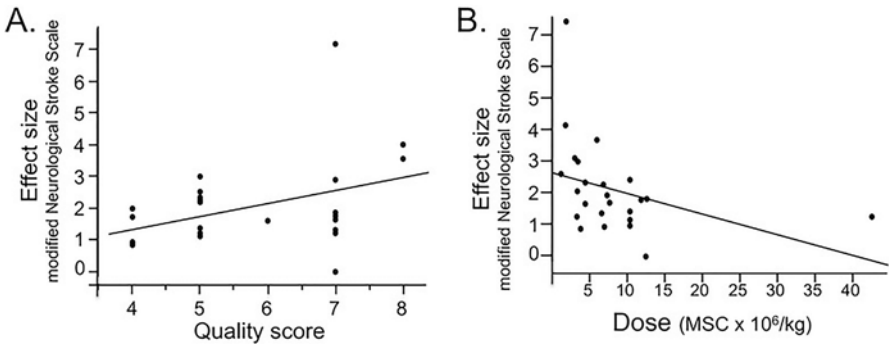

Fig. 17.3 For preclinical studies that introduced MSC ≥ 24 h post-stroke: (**a**) Higher Quality Score was associated with greater behavioral effects of MSC ($r = 0.42$, $p < 0.04$), and (**b**) Lower MSC doses were associated with greater behavioral effects ($r = -0.58$, $p < 0.002$). From Vu et al. [89], with permission

In sum, this meta-analysis examined preclinical studies of MSC in the treatment of ischemic stroke. The main finding was that a significant favorable effect of MSC was observed in 44 of the 46 studies. Effect sizes were large, remained substantial after adjusting for potential publication bias, and were robust across species, delivery route, time of administration in relation to stroke, MSC immunogenicity, and MSC dose. A second key finding was that higher study quality was associated with larger behavioral gains after MSC administration (Fig. 17.3a). This finding contrasts with the more common pattern whereby lower quality studies overestimate intervention effects [94–99] and encourages translational efforts.

Treatment of stroke focuses on separate strategies according to the time post-injury, with very early interventions focusing on tissue salvage, and later interventions targeting neural repair. These results emphasize that this distinction remains important in the use of MSC to treat stroke. The effect on infarct volume reduction was highest when MSC were initiated at the earliest times (0–8 h after stroke onset, Fig. 17.2b), consistent with an acute neuroprotective MSC effect. Conversely, MSC introduced ≥24 h post-stroke had a large effect on behavioral recovery. Translational studies of MSC after stroke need to carefully consider therapeutic targets in relation to respective time windows.

Clinical Trials of MSC in Human Subjects with Stroke

MSC have an overall excellent safety record in clinical trials of human subjects across numerous non-cerebrovascular diagnoses [25, 100–103]. Results from the limited number of studies to date examining MSC in patients with stroke are consistent, suggesting no safety concerns. As with preclinical studies, the preponderance

of investigations has been on repair rather than on acute neuroprotection, and on ischemic stroke, with limited data on hemorrhagic stroke.

Initial human stroke trials focused on autologous MSC. Bang et al. [104] randomized patients with ischemic stroke into control and experimental groups, with the experimental groups receiving IV infusion of 10^8 autologous BM–MSC 4–9 weeks following onset of symptoms. MSC therapy was associated with significantly improved functional status (modified Rankin score and Barthel index) up to 6 months after transplantation, as compared to controls. At follow-up 5 years later, functional gains were sustained [105], and no MSC-related effect on mortality or malignancy was reported. Honmou et al. [106] cultured MSC in autologous human serum rather than fetal calf serum, reducing cell preparation time as well as any potential risk of transmissible disorders. Patients received IV autologous MSC 36–133 days post-stroke, using a study design that did not include a control group. MSC administration was not associated with adverse events or neurological deterioration. Bhasin et al. [107] found that IV transplantation of autologous MSC in six patients with chronic (3–12 months following) stroke was safe as compared to six controls and that MSC treatment was not associated with any significant change in neurological function.

Subsequent efforts have extended to allogeneic MSC. Steinberg et al. [108] reported the results of an early phase open-label study, "A Novel Phase 1/2A Study of Intraparenchymal Transplantation of Human Modified Bone Marrow Derived Cells in Patients with Stable Ischemic Stroke," sponsored by SanBio. Patients were 6–60 months after an ischemic stroke in the territory of the middle cerebral artery (MCA), with moderate to moderately severe disability [modified Rankin scale (mRS) score 3 or 4]. A total of 18 patients received one of three escalating cell doses (2.5, 5, or 10 million), intracerebrally, at one of two US sites. The cells were adult bone marrow-derived MSC with transient Notch transfection. The primary endpoint was safety, with 2-year follow-up, and no concerns were identified. A number of secondary clinical and imaging endpoints were examined, with some behavioral measures showing improvement at 6 months following surgery. A small number of subjects showed substantial, very rapid behavioral improvement that may have been associated with appearance of new T2-bright areas on MRI.

Yavagal et al. [109] reported the results of the RECOVER-Stroke trial, sponsored by Aldagen. The primary objective focused on the safety of intracarotid (IC) infusion of ALD-401 cells in patients with subacute anterior circulation ischemic stroke. This was a phase 2A, double-blind, randomized, sham-controlled trial of 48 patients who were 13–19 days after an ischemic stroke in the territory of the MCA or anterior cerebral artery. Other key entry criteria included age 30–75 years, mRS score ≥ 3 at time of randomization, and patent ipsilesional carotid artery, with patients having pre-stroke disability or severe medical comorbidities excluded. Across eight US sites, all patients received IC injection, the content of which was randomized 3:2 (cells:sham). The cells were ALDHbr, which are isolated from autologous bone marrow and selected based on high expression of aldehyde dehydrogenase, transfused at a mean total dose of 3.1 million cells (0.2–7.4 million

cells). Over 1 year of follow-up, no safety concerns were identified. Secondary analyses did not identify any differences between the two treatment groups in change in various behavioral measures.

Hess et al. [110] reported the results of a double-blind, placebo-controlled, dose escalation study of MultiStem in patients with acute stroke, sponsored by Athersys. The primary objective of the study focused on two primary endpoints, safety and efficacy. Cells were MultiStem, a biologic product of adherent progenitor cells depleted of CD45(+) cells derived from adult bone marrow or other non-embryonic tissue sources, and were transfused IV 24–48 h after an ischemic stroke affecting cerebral cortex, at one of 33 sites in the USA or UK. Patients had baseline NIHSS score 8–20 that was stable, and lacked significant medical comorbidities. Three doses were studied, up to 1.2 billion cells. Over 1 year of follow-up, there were no safety concerns, defined in terms of frequency of dose-limiting adverse events. However, there was also no difference between the two treatment arms in efficacy, defined as stroke recovery to day 90 based on a global test analysis that included mRS, NIHSS, and Barthel Index. Some secondary analyses suggested reduced short-term mortality in the Multistem arm, and also the possibility of greater Multistem effects on stroke recovery when administered earlier (<36 h post-stroke). The authors also noted that patients receiving Multistem showed significantly reduced rates of circulating CD3+ T-cells 2 days after transfusion.

Several guidelines have been published that inform translation of MSC to human clinical trials, including those from the Stem Cell Therapy as an Emerging Paradigm for Stroke (STEPS) committee [6, 111, 112]. This group provided a series of recommendations regarding preclinical and clinical research into stem cell-based therapies for ischemic stroke. Some recommendations echoed the STAIR recommendations, which describe issues of stroke clinical trial design in broad terms [113]. Key issues include structuring entry criteria with respect to properties of the cell therapy of interest, the natural history of the stroke, and study end points; choosing a time window for patient selection that is based on preclinical findings; consideration of modality-specific endpoints [114], which are sensitive to the differences in recovery within individual neural systems; and attention to rehabilitation dose, which is a covariate in restorative studies.

Principles of Brain Repair and MSC Therapy After Stroke

Most research into MSC therapy after stroke has focused on restorative time windows and biological targets. A number of principles pertain to neural repair therapies [115, 116].

First, brain repair is time sensitive [117]. Some biological targets are only relevant during a specific time period after stroke [29, 118, 119], and some therapies have different effects depending on time of administration post-stroke [120–126]. Some data suggest that behavioral gains from MSC administration are greater when therapy is initiated 7 days after stroke onset as compared to 1 day after stroke onset [89].

Second, brain repair is experience dependent. Since the classic studies by Feeney et al. [127], which showed that a stimulant improved motor outcome only when paired with training, increasing evidence suggests that a restorative therapy needs the right kind of experience to produce best results [128–132]. A critical threshold of post-stroke rehabilitation has been found below which BDNF levels do not increase. The need to pair a plasticity-receptive brain with relevant behavioral experience is reminiscent of the critical periods of normal brain development [133] and emphasizes the parallels between development and recovery [134]. The experience-dependent nature of repair-based therapies is in contradistinction to acute neuroprotection-based or acute reperfusion-based therapies; for example, subjects receiving IV tPA are not asked to engage in any particular training paradigm to maximize drug effects. Introducing a therapy such as MSC that promotes brain plasticity sets the stage for improved outcomes, but available data suggest that treatment effects are likely maximal when appropriate training and experience are provided in parallel.

Third, patient selection and stratification are critically important to post-stroke brain repair after stroke using MSC. Stroke is extremely heterogeneous, as patients differ in pre-stroke status, nature of stroke injury, post-stroke deficits and recovery, therapies provided, concomitant comorbidities, and more. The issue was well described by Bath et al., who noted that "In stroke trials, the impact of covariates such as age and severity on outcome is typically much larger than the treatment effect that is being measured" [135]. Enrolling and analyzing patients with attention to key variables can reduce variance and increase statistical power, enabling a study to detect a treatment effect in the appropriate population when such an effect is indeed present. Numerous variables have been found to be potential predictors of stroke outcome, including location and size of injury [136–138], genotype [139–141], measures of brain function [136, 138, 142], and degree of depression [143, 144]. Such measures may be of pivotal value in defining the population most likely to benefit from a given therapy. This point was illustrated in the analysis of a recent Phase III restorative stroke trial of cortical stimulation, which failed to detect a difference in motor outcome between active and control groups [145]. Each of the preclinical studies in rodents and primates required preserved physiological integrity of the motor system, but the clinical trial did not. A post hoc review [146] of patients randomized to active therapy (stimulation) found that the treatment effect was significantly greater in those patients who had preserved physiological integrity of the motor system. These findings suggest that preserved physiological integrity may be a useful entry criterion in such investigations and furthermore emphasize the need to design translational stroke clinical trials with key features of preclinical studies in mind.

Fourth, modality-specific measures might be useful to measure treatment effects [114]. The effect of restorative therapies is most apparent in those neural systems that have sustained sub-maximal injury; neural systems that are utterly ablated by injury may lack sufficient substrate to improve, and systems that sustain only mild injury may have a ceiling effect in terms of showing treatment-induced gains. A specific behavior for which the neural underpinnings are sub-maximally

injured—such as motor function or language—might show substantial gains in response to a restorative therapy. In such an instance, behavioral gains are likely to be captured with modality-specific endpoints, such as those focused on motor or language function. In contrast, outcome measures focused on global behavioral status, such as the NIHSS or mRS, may lack the granularity to detect system-specific behavioral gains.

Summary

MSC are a form of multipotent stem cells that have multiple mechanisms of action and are relatively immunoprivileged. Abundant preclinical evidence supports their efficacy in stroke, across species, delivery route, time of administration in relation to stroke, MSC immunogenicity, and MSC dose. Initial human studies support the safety of these cells. A number of issues exist in relation to MSC manufacture and are likely to receive increasing attention in future human clinical trials. The design of clinical trials examining MSC after stroke can benefit from consideration of several principles of brain repair.

Disclosures Steven C. Cramer has served as a consultant to GlaxoSmithKline, Microtransponder, Dart Neuroscience, RAND Corporation, and Roche. He is cofounder of personalRN.

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Chapter 18
Glioma Stem Cells

Regina Teresa Martuscello, Brent A. Reynolds, and Santosh Kesari

Abstract The acknowledgment of active stem cells within the adult CNS and the subsequent association of cancer etiology have brought about an entirely new field of study. Cancer stem cells (CSCs) have gained significant traction in oncology research with the discovery of a treatment-resistant, highly tumorigenic subpopulation of tumor cells. Multiple theories exist as to the cellular origins of CSCs and their abilities to differentiate from precursor cells to solid tissue malignancies. Each of these theories and the following stem cell hypotheses relating to the differentiation and cellular distribution capabilities of stem cells will be discussed. In gliomas, the search for the glioma stem cell (GSC) has brought about numerous researchers looking to identify external markers for GSC classification. To date, there has been no successful identification of a GSC within any patient or immortalized cell line by external marker. Here, we will also discuss the research done in search of a GSC marker and the consequent possible treatment options for cells that have been identified as highly tumorigenic, treatment resistant, and metastatic.

Keywords Brain cancer • Central nervous system tumor • Gliomas • Stem cells • Glioblastoma • Cancer stem cells • Astrocytoma • Targeted therapy • Immunotherapy

Introduction

The human brain is composed of diverse cell types numbering in the millions of units. Nerve cells, or neurons, are responsible for the electrochemical signaling required for all higher functions found in humans. Satellite cells, or neuroglial cells, comprise about one-half of the volume of the brain, greatly outnumbering neurons.

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Glial cells are separated into three classes: oligodendrocytes, astrocytes, and radial glial cells [1]. Uncontrolled aberrant cellular growth derived from glial cells in the brain results in glioma formation. Glioma is used as an umbrella term for tumors developing from any subclass of glial cell and can be broken down further into World Health Organization (WHO) grading subclassifications (Table 18.1) [2]. Approximately, 80 % of all diagnosed malignant and benign brain tumors are

Table 18.1 WHO grades of CNS tumors

Tumor classification	Tumor grade (WHO)
Astrocytic tumors	
Pilocytic astrocytoma	I
Diffuse astrocytoma	II
Anaplastic astrocytoma	III
Glioblastoma	IV
Oligodendroglial and oligoastrocytic tumors	
Oligodendroglioma	II
Anaplastic oligodendroglioma	III
Oligoastrocytoma	II
Anaplastic oligoastrocytoma	III
Glioblastoma with oligodendroglioma component	IV
Ependymal tumors	
Subependymoma	I
Myxopapillary ependymoma	I
Ependymoma	II
Anaplastic ependymoma	III
Choroid plexus tumors	
Choroid plexus papilloma	I
Choroid plexus carcinoma	III
Neuronal and mixed neuronal–glial tumors	
Ganglioglioma	I or II
Central neurocytoma	II
Filum terminale paraganglioma	I
Dysembryoplastic neuroepithelial tumor (DNET)	I
Pineal parenchymal tumors	
Pineocytoma	II
Pineoblastoma	IV
Embryonal tumors	
Medulloblastoma	IV
Supratentorial primitive neuroectodermal tumor (PNET)	IV
Atypical teratoid/rhabdoid tumor	IV
Meningeal tumors	
Meningioma	I
Atypical, clear cell, chordoid	II
Rhabdoid, papillary, or anaplastic (malignant)	III

gliomas, and upwards of 30 % of those are glioblastoma (grade IV). This is largely due to the absence of early detection systems, relying solely on patient reported neurocognitive impairments, resulting in late-stage diagnoses. Clinical manifestations of tumor formation are varying with generic symptoms such as seizures, focal weakness, nausea, blurred vision, and speech or memory impairment. The primary method of diagnosis and tumor grading is through histopathological analysis of patient tumor biopsies. Advancements in state-of-the-art imagining for analogs such as glucose uptake (fluoro-deoxy-glucose/FDG-PET) and proliferation (fluoro-thymidine/FLT-PET) are enabling better assessment of tumor infiltration and treatment outcomes. However, because gliomas are composed of a group of heterogeneous cell types that can express an assortment of neural lineage markers, the subsequent tumors share similar morphology and phenotype, yet have diverse prognosis and treatment responses.

Cerebellar Neuron and Glia Development

The developing cerebellum holds two distinct germinal regions: the external germinal layer (EGL) contains committed granule cell precursors (GCPs) that only generate granule neurons and the ventricular zone (VZ) contains multipotent stem cells that give rise to the bulk of cerebellar neurons and glia [3]. There are both neural stem cells and glial progenitor cells in various regions of the human adult brain. Neural stem cells have been isolated from the subventricular zone (SVZ), the lining of the lateral ventricles, within the hippocampus, the dentate gyrus, and the subcortical white matter. In humans, it has been shown that the SVZ holds a population of astrocytes that can serve as neural stem cells. In other adult mammals it has been shown that glial progenitor cells throughout the neuraxis are capable of producing astrocytes and oligodendrocytes. These stem cell and progenitor elements, along with differentiated adult glia, represent a faction for neoplastic alteration [4]. One potential consequence of this active adult neurogenesis implies that a debility or defect in the process may play a part in glioma formation [5].

Cell of Origin in Glioma

The derivation of tumor initiating cells from normal or immature neural cells and the subsequent process that acquires limitless self-renewing and replicative abilities of these cells are still unknown and under debate. Six major alterations are required for cancer progression: self-sufficiency in growth signals, limitless replicative potential, sustained angiogenesis, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), tissue invasion, and metastasis [6]. The ability for cancer cells to gain these diverse functionalities as a solid entity hints to a stem-like developmental cell of origin. However, it is essential to

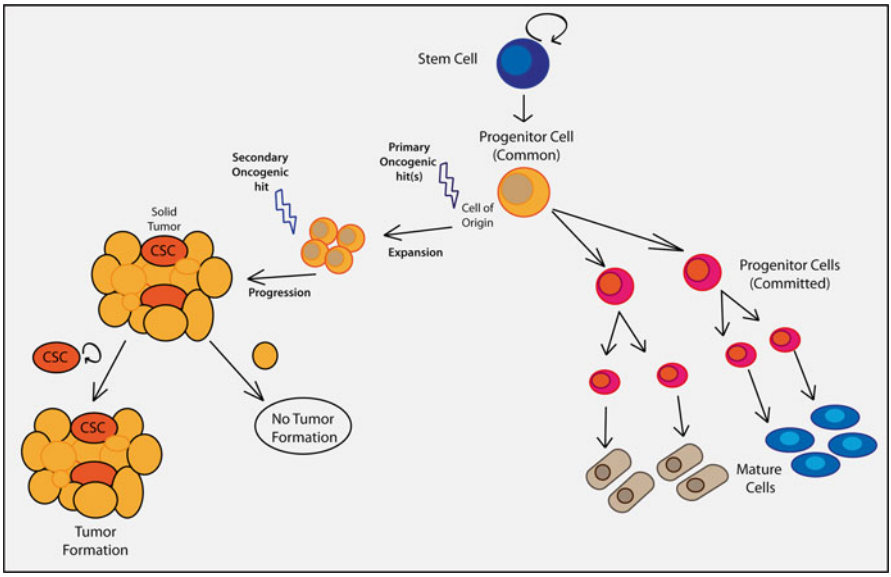

Fig. 18.1 The evolution of a cancer stem cell and the hierarchy distinction from the cell of origin. Stem cells produce all mature cell types for specific tissues from the progressive generation of diverse progenitor cells, which can be common giving rise to more committed progenitor cells. Under normal developmental cellular hierarchy the stem cell sits on top of this cascade, constantly undergoing self-renewal. The cell of origin for an individual tumor could be an early precursor cell, such as a common progenitor cell, created by a stem cell. The resulting cellular population then accumulates increasing amounts of epigenetic mutations causing an abnormal population of expanding cells undergoing neoplastic progression resulting in the emergence of a cancer stem cell (CSC). In this model of tumorigenesis, only the CSCs are capable of sustaining malignant growth. Therefore, the cell of origin may be distinct from the CSC, which spreads the tumor

make the distinction that the cell of origin is not automatically the cancer stem cell (CSC). The cell of origin would be a normal cell that develops the initial cancer-promoting mutations and results in tumor initiation, whereby the CSC would be the subset of cells within the tumor that uniquely maintains malignant growth. This also differs from the cell of mutation, which is the cell type that gains the primary oncogenic alterations but does not necessarily proliferate until another point in its respective cellular order [7] (Fig. 18.1). It is unclear if more than one cell of origin or cell of mutation exists for a single tumor type. There have been three theories as to how the cell of origin can give rise to high-grade gliomas (HHG): the *de-differentiation theory* deems tumorigenesis as a multistep progression accompanied by genetic aberrations of a normal cell, resulting in progressive cellular transformation of highly malignant cells, CSCs. This theory stems from the observation that the expression levels of differentiation markers within HHG are lost compared to low-grade gliomas (LGG). Animal models have given further support for this theory, as the activation of specific oncogenes paralleled with a loss of tumor suppressors in cortical astrocytes prompts cancer induction with similar histological features to

HGG. The *precursor cell theory* states that neural precursor cells, such as oligodendrocyte precursor cells (OPCs) and NG2+ (glial precursor) cells could be the cell of origin in HGG [5]. OPCs are very active within the adult brain and their inherent plasticity allows them to be converted in vitro into immature multipotent cells, which are able to give rise to numerous glial and neuronal cell lineages. HGGs freely express markers associated with OPCs, such as NG2 and PDGFR, whereby the PDGFR-alpha signaling pathway controls proliferation and migration of OPCs and is commonly altered in glioblastoma (GBM). The final theory is the oldest, first being introduced by Virchow in 1863. The *stem cell theory* proposes that tumors originate from dormant or quiescent cells located somewhere within the host tissue. This theory is based on the histological similarities seen between embryonic stem cells and cancer cells. In the late 1990s, this theory took hold when several groups demonstrated that many cancers were composed of highly tumorigenic cells that not only displayed many of the classic stem cell features, but also were able to generate complex tumor formation upon transplantation. However, it is clear that these theories, while distinct, are not mutually exclusive. Rather, it is most likely that a combination of changes in things such as cellular microenvironment, host environment, metabolomics, and epigenetics cause an amalgamation of the three [8]. This has initiated multiple stem cell hypotheses to be proposed, taking leads from leukemia and hematopoietic stem cell research. Chromic myeloid leukemia (CML) and acute myelogenous leukemia (AML) are just two examples of hematopoietic malignancies that result from a specific subset of genetically aberrant stem cells.

Stem Cell Hypotheses

At the time of diagnosis, tumors are composed of cellular heterogeneous clones in both genetics and phenotypes. Intratumoral heterogeneity has traditionally been viewed according to a stochastic model [also known as the clonal model]. This model explains cancer heterogeneity as an evolutionary growth process, whereby malignancy arises from a single cell of origin. The resulting tumor progression stems from a random collection of somatic mutations in a genetically unstable cell population, while consecutive selection pressures from environmental cues trigger neoplastic changes into subclones (Fig. 18.2a) [9]. In accordance with this model, it has been postulated that individual-specific anticancer therapy may be required due to the large number of potentially random mutations that might drive tumor growth. Furthermore, the subsequent emergence of treatment resistant subclones from neoplastic cells with equal or heightened tumorigenic potential may always evade therapy. Although clonal heterogeneity has been extensively documented in gliomas and many other cancers, accruing evidence suggests a secondary level of functional heterogeneity that exists based upon this cellular differentiation. Functional heterogeneity within cancer was shown decades ago by the fact that only minor subsets of cells within a tumor are capable of clonogenic growth in mice or culture. In opposition to the stochastic model, the cancer stem cell (CSC) model [also know as the

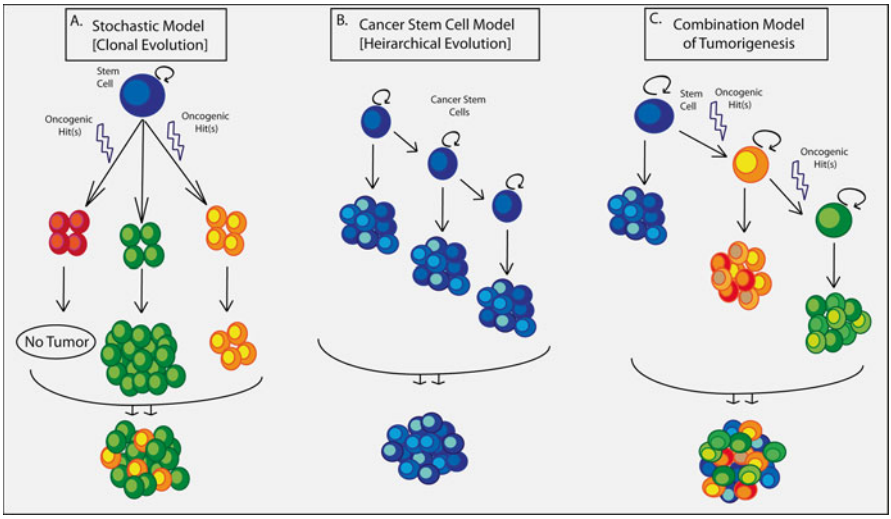

Fig. 18.2 Models of cellular cancer heterogeneity.
(**a**) The stochastic, or clonal, model of tumorigenesis assumes that cancer cell phenotypes are principally defined by the inherent factors gained through driver mutations. These mutations give rise to clonal evolution of cancer cells whereby some cells can give rise to solid tumors and others cannot. This model, while explains the cellular heterogeneity, does not address phenotypic variations found within individual cellular clones. (**b**) The cancer stem cell model of tumorigenesis; the tumorigenic potential is limited to the cancer stem cell population. This model assumes that the malignant growth is organized via hierarchical evolution and hypothetically resembles the tissue of origin. Cellular heterogeneity of the tumor is then a result of multipotent cancer stem cells. This model does not effectively address the sustained maintenance of coexisting genetically diverse clones in most late-stage cancers. (**c**) It has been proposed that perhaps a combination of these two models may be most likely occurring, whereby, one or more dominating clones, some of which may be organized in a hierarchical manner, drive cancers. However, it is always possible that the acquisition of genetic or epigenetic mutations may promote tumorigenic capacity and impair differentiation at any point in the evolution of cancer cells

hierarchical model] suggests a categorized organization of functional heterogeneity, with self-sustaining CSCs at the top, giving rise to heterogeneous trans-amplifying differentiated cancer cell types (Fig. 18.2b). As with the debate on the cell of origin in glioma, the stochastic and cancer stem cell models interpret intratumoral heterogeneity differently, yet again it is significant to note that the two are not mutually exclusive. Significant genetic incidents constantly accumulate in CSCs and their progenies, which progressively give rise to new genetically discernible daughter cells. The structure of these new cells may or may not be hierarchically organized. Consequently, the wide varieties of document CSC phenotypes may reflect the overwhelming complexities of cancer genomes. While direct experimental evidence is still emerging, it has been hypothesized that the cellular complexity of many human cancers is likely the result of some combination of hierarchical differentiation and clonal genetic events (Fig. 18.1c) [10].

This complexity continues, as the ability to ascertain which type of solid tumor arises from transformed stem cells and identification of the corresponding tumor-initiating stem cells are major challenges. These points are of special concern because cancers derived from stem cells would have a more diverse and invasive phenotype than would those derived from more restricted progenitors. Therefore, the cellular heterogeneity found in most cancers could be generated not only by genetic instability and epigenetic changes, but also by the aberrant differentiation of cancer stem cells and their cellular divisions. Cells can divide either symmetrically or asymmetrically. Unlike conventional symmetrical mitosis, which generates two identical daughter cells, asymmetrical cell division leads to the separation of disproportionate cell-fate determinants. Therefore, the two produced daughter cells will have diverse cellular outcomes. For example, a stem cell that has undergone asymmetrical division gives rise to a self-renewing stem cell and to a daughter cell. This daughter cell is the progenitor, which engages in the differentiation process, and has a different programmed cell fate. Yet, two daughter cells produced by symmetrical division can also acquire different fates as a result of their exposure to diverse environmental influences. This point is of great importance for cancer research, as the field moves toward the notion that tumors have a stem cell of origin. Interestingly, asymmetrical cellular division would not increase the pool of CSCs, as it will only generate one CSC and one progenitor cell. In accordance with this model, it is the symmetrical division of the progenitor that constitutes the expanding pool of tumor cells. An immediate consequence of this situation is that if tumor progenitors migrate away from the site where cancer stem cells reside, then tumor lesions and cancer stem cells may not necessarily match each other in location. In gliomas, this asymmetrical division of malignant neural stem cells is then expected to produce two different daughter cells: one that remains in the germinal zone as a cancer neural stem cell and one that migrates away and proliferates as a cancer neural progenitor. One potential effect of this asymmetrical cell division is that the intrinsic chemosensitivity of the two different daughter cells might differ. Another important outcome is that the tumor does not reside in the same site as the malignant neural stem cell, but at the location where the proliferating tumor progenitors may have migrated. This shows the increased complexity when trying to identify the intra- and extracellular environmental cues that specify whether a cancer stem cell will undergo symmetrical or asymmetrical divisions [11, 12]. Finally, with the knowledge that in leukemia there is a population of self-renewing, infrequent cycling, cancer stem-like cells, Deleyrolle et al. looked into the division rate of glioblastoma cancer cells. They showed that there are indeed two populations of glioma cancer cells that are cycling either at a faster or slower rate. By loading cells with CSFE [a dye that loses fluorescence upon every cell division], they can identify populations of cells that are dividing frequently or infrequently. Their data effectively shows that label-retaining cells, defined as slow-cycling fractions, exist within human gliomas and that this population of cells are enriched in tumor initiation cells expressing stem-cell markers and exhibit functional characteristics of tumor stem cells in culture and in limiting dilution transplantation assays [13].

Characteristics of a Stem Cell

One of the most important characteristics for human tissue homeostasis is the ability for continuous cellular turnover to produce terminally differentiated and mature cells. This is a vital process that allows for human embryonic development, adult organ function, and the ability for the body to repair after injury. In order to accomplish this there is a large degree of flexibility required in the genesis of new cells as to compensate for the expansive oscillations in cellular physiology [14]. This homeostatic system requires a complex, sequential lineage in which highly undifferentiated cells, capable of extensive growth and differentiation capacity can give rise to progeny that can narrow their abilities to ultimately obtain structural and functional characteristics of their home tissue. At the apex of this cellular hierarchy is the stem cell, which induces a series of progenitor cells that progressively lose their capacity for extensive, independent self-maintenance. Therefore, an essential concept in stem cell biology deems that the most dependable way to characterize and categorize different neogenic cells is to identify them according to their specific role and fundamental properties within a tissue [15]. Whereby a stem cell can be identified through a set of features, which would provide an unequivocal stem cell definition; Potten and Loeffler did this in 1990 [16]. The five original detailed characteristics have been expanded and applied to current stem cell knowledge and are shown in Fig. 18.3 [left]. The ability to identify these characteristics in stem cells has been progressively studied and confirmed in the 20 plus years since its debut. The ability to apply and identify these characteristics to CSCs has been a relative challenge and has altered a few of the defining characteristics with in the Potten and Loeffler table (Fig. 18.3 [right]). Therefore, it is important to note that there are challenges in the current technological ability to identify each of these

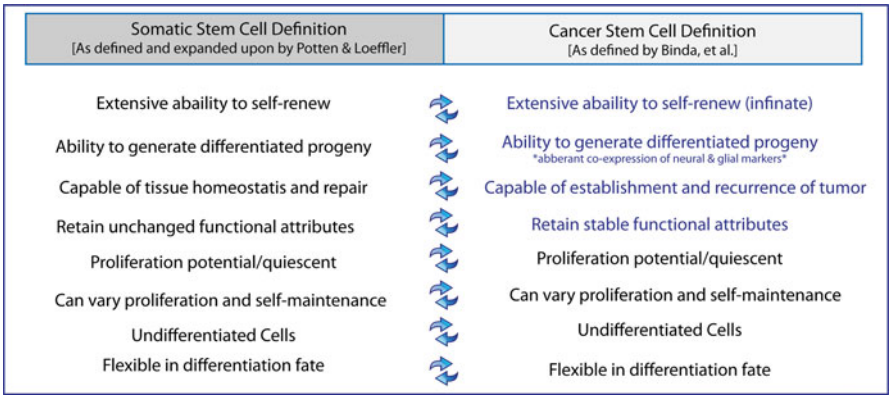

Fig. 18.3 Comparison of defining criteria of somatic stem cells and cancer stem cells. *Left*: Derived and expanded upon from the classical work of Potten and Loeffler; an operational definition of somatic stem cells. *Right*: The proposed definitions of cancer stem cells (CSCs) as derived from that of their normal counterparts. The most critical functional characteristics are highlighted in blue and should be possessed by a candidate CSC

characteristics within a CSC. Consequently, it is acceptable to distinguish stem cells based on some of their more highly specific characteristics, particularly the ability to maintain self-renewal, differentiate, and repair after injury (Fig. 18.3 [blue]). This operational definition then relies on the functional assessment of the cell candidate properties, causing stem cell identification to then be retrospective. Armed with this knowledge, it will then be important to try and identify potential CSCs candidates within a pool of tumor cells through external identification and subsequent testing of cellular functional features.

Identification of CSCs

To date, no single antigen has been shown to reliably segregate tumorigenic stem cells from the rest of the tumor cell population in gliomas. Other cancer types such as prostate, blood, breast, skin, and colon have shown the existence of a highly tumorigenic and slow cycling cell fraction. The existence of CSCs in gliomas and their true origins cannot be fully elucidated until there are reliable methods of identification of a cellular subpopulation of highly tumorigenic stem cells. Although several markers have been identified and may be informative toward brain tumor stem cell identification, the ability to distinguish a normal stem cell from a cancer stem cell as well as a stem cell from a progenitor is still inadequate [17–19]. Furthermore, the ability to identify the cell of origin, from the cell of mutation, from the progenitor cell will require enhanced experimental techniques that follow the patient-specific evolution of brain tumors. One issue in the identification of CSCs is the lack of continuity in the culture of cancer cells. There is still debate as to whether cultures grown in monolayer as opposed to spheroid suspension better reproduce clinical tumor outcomes. The original assay that identified neural stem cells [neurosphere assay (NSA)] supplements cells with growth factors rather than serum and provided the ground work for breaking the no new neuron dogma, which stated that there was no new adult neurogenesis. Tumor samples removed from patients and exposed to the NSA provided the first selective pressure assay that resulted in only a subset of cells having spheroid abilities [20]. It was proposed that cells, which have the ability to form spheres, represent the stem-like-cell fraction of tumor cells. Upon analysis of glioma cells grown as spheres (gliomaspheres), it was found that these cells better recapitulate the phenotypic, genotypic, and histological characteristics of clinical patient tumor samples [21]. Furthermore, artificial culture methods of malignant cells result in the continuous accruement of divisional mutations, causing tumor cell characteristics far separated from the host. With the emergence of the NSA, researchers were looking to identify a marker or antigen specific to sphere-forming cells compared to non-sphere forming cells. The ability to identify a cancer stem cell by an external marker could have profound implications in the current therapeutic strategy of brain tumors as the CSC is the highly tumorigenic cell capable of evading current anti-cancer therapies.

CD133

Since the discovery of the CD34+/CD38−hematopoietic stem cell marker, researchers have been striving to find the stem cell marker for gliomas. CD133 is a five-transmembrane glycoprotein located in the membrane of human hematopoietic cells and in neural progenitor cells. CD133 is currently considered a stem cell marker in a diverse set of normal tissues, as well as different cancer types (leukemia [22], prostate cancer [23], colon cancer [24], lung cancer [25], hepatocellular carcinoma [26], ependymoma [27], melanoma [28], ovarian cancer [29], medulloblastoma [30], and glioblastoma [1, 31]). Several studies have shown that CD133 has the ability to enrich populations of cells with stem-like features. However, there is currently a growing body of evidence that is limiting this observation and restricting CD133 as a potent stem cell marker [19]. The expression of CD133 has been discovered in prostatic epithelial and neural stem cells, endothelial progenitors, and myogenic cells. Using cells isolated postmortem from fetal and adult human brains, researchers isolate the CD133+ fraction of cells using FACS and underwent orthotopic transplantation in the brains of mice. Cells transplanted show extensive self-renewal potential and had the capacity to not only engraft and migrate but to also undergo neural and glial cell differentiation. In gliomas, Singh et al. were the first to describe the CD133+ tumor cell population, citing the stem cell characteristics of self-renewal and the ability to histologically reproduce the original tumor in the brains of immunocompromised mice. In addition, they demonstrated that they could produce a tumor with as few as 100 CD133+ cells, whereby the injection of more than 1×10^5 CD133− cells did not produce a tumor. In Singh et al., and other publications, it was shown by quantitative FACS analysis that the CD133+ percentage of cells within the population was low and many times barely detectable. This was seen across human gliomas, gliomasphere cultures, and established cell lines, giving support to the assumption that CSCs are a rare population of cells in a solid tumor mass. However, other studies have shown that rather than a rare population of cells, they see an exceptionally high CD133+ fraction of cells, sometimes reaching 50–60 %. Immunohistochemical analysis of human glioma samples find that many tumor samples contain more than 25 % of CD133+ cells [32]. Patient studies looking into the prognostic value of CD133+ have also reported inconsistent finding, with some groups finding both a positive quantitative correlation with glioma grade and a negative association with patient survival. Four independent groups found CD133 as a negative prognostic factor in survival with astrocytoma patients [33–36]. Yet a different group [37] found no correlation with CD133+ expression in either tumor grade or clinical outcome. Other research groups have attempted to replicate the transplantation assay of CD133+ and CD133− cells with mixed results, showing tumor growth now in both populations. It is notable that CD133 antigen can be used to enrich for CSCs through flow cytometry; however, it will require further analysis as to whether or not this expression can be measured by mRNA and/or protein to identify CSCs. However, there is more evidence to suggest that CD133 positivity is not the deciding stem cell factor. It has been shown that numerous GBM

samples do not contain detectable CD133. It has also been shown that many established glioma cell lines do not carry CD133, yet are able to produce solid tumors upon transplantation. As well, the ability for glioma cells to form spheres in culture is irrelevant to CD133 status, showing stem cell properties of self-renewal and propagation of tumor formation. Taken together it is clear that there is a division in the CSCs with respect to CD133 positivity, especially in glioma. This evidence suggests that there is the existence of both a CD133+ and CD133− glioma stem cell population [38]. Taking this into account, one such study looked into the gene expression profiles of CSCs isolated from CD133+ and CD133− gliomas [39]. Their findings led them to define two different populations of glioma stem cells: type 1 cancer stem cells, which are CD133+ and grow as gliomaspheres and resemble fetal neural stem cells and type 2 cancer stem cells, which grow in adherent culture and resemble adult neural stem cells. These finding have also had inconsistency in the literature showing that both CD133+ and CD1133− cells isolated from the same tumor specimen can be cultured as neurospheres and both populations are able to self-renew and initiate tumor formation. It is clear that CD133− cells are capable of not only generating CD133+ cells in vitro and in vivo, but are capable of driving tumorigenesis equal to CD133+ cells. Trying to make sense of the convoluted positivity and negativity of CD133 has been a challenge.

In 2010, Chen et al. endeavored to understand the CD133 antigen in glioma stem cells and looked to identify the positive and negative cell characteristics to create a functional hierarchy. This group attempted to demonstrate the existence of three different, yet coexisting, types of glioma stem cells: type 1 is a CD133− cell that is able to generate CD133+ progeny, type 2 is a CD133+ cell able to generate CD133− progeny and type 3 is a CD133− cell that can only generate CD133− progeny. This segregation of CD133 status of parent and progeny has established a hierarchical lineage between these three types of cells, suggesting the cellular CD133− status is a nascent cell that gives rise highly tumorigenic CD133+ cells in gliomas [40]. Further research may shed new light onto the CD133 debate.

L1CAM and CD133+ Cells

L1CAM is a neuronal adhesion molecule that is necessary for sustaining the proliferation and survival of CD133+ glioma cells. During development of the central nervous system, L1CAM has been shown to regulate neural cell survival, growth, and migration. However, its role in the adult central nervous system is unclear. In glioma, L1CAM has shown to be overexpressed and play a role in tumor invasion [41, 42]. L1CAM+/CD133+ glioma cells have extensive interaction in vivo and in vitro, showing cosegregation and increased expression levels in glioma cells than in normal neural progenitor cells. In vitro, the lentiviral targeting of L1CAM using short hairpin RNA (shRNA) knockdown in CD133+ glioma cells resulted in inhibition of not only cellular growth but also sphere forming ability of glioma stem cells

and rather induced apoptosis [43]. To date, it has not been shown whether or not L1CAM positive or negative expression levels in glioma cells have any correlation with tumorigenic potential.

CD15

CD15 or SSEA-1 (stage-specific embryonic antigen-1) is a trisaccharide and has been shown to be expressed in adult neural stem and progenitor cells as well as in embryonic stem cells during neural development. In the ongoing search for external stem cell markers, multiple groups have identified CD15 as a selectively expressed surface protein in cells with tumorigenic capabilities. In medulloblastoma, it was demonstrated that there is a distinct population of cells that express CD15 able to produce a tumor in a mouse model. In this model, CD133 expression was variable and was not specifically localized within the stem cell compartments [44]. In glioblastoma, it was shown that CD15 could act as an enrichment marker for stem cells in CD133− tumors. Whereby human GBM specimens that were found to be completely CD133− and then selected for CD15 positivity resulted in the capacity to form colonies and neurospheres in culture, differentiate into glial and neuronal marker expressing cells and be highly tumorigenic upon serial transplantation in vivo [45]. Additionally, there has been an established ordered heredity between CD15+ and CD15− cells, whereby CD15+ cells have the unique capability to generate the cellular heterogeneity of the initial patient tumor. Yet, as is the case with CD133, CD15 expression is widely variable in patient GBM samples, and its ability to enrich for a stem cell population does not work with every tumor.

CD44/Id1

Several groups have identified CD44 as a cell surface marker for CSCs in breast, pancreas, and prostate cancers [46–48]. In glioma, only one group looking into glioblastoma, the classic example of HGG, found CD44 and Id1 to be stem cell markers [49]. Ids (inhibitors of DNA-binding proteins) are transcription factors that antagonize the DNA-binding capacity of basic helix–loop–helix factors and regulate cell cycle and cell differentiation, playing an important role in stem cell self-renewal. Specifically, Id1 has shown to be expressed in B1 type adult neural stem cells, and in cancer Id1 it has been shown to be upregulated in several tumor types and has been described as being potentially involved in metastasis. In endothelial niches of tumor tissue, Anido et al. showed that CD44high/Id1high cells were localized and possessing stem cell characteristics. They also showed that this high expression level of CD44 and Id1 is inversely correlated with patient survival and prognosis. However, as in the case with all previous stem cell markers, there is a disconnection between marker expression and tumorigenic potential. While there is

correlation between Id1 expression and stemness in vitro, in vivo Id1 low expressing cells can extensively expand upon transplant. Further research into these stem cell markers is needed to fully understand the relationship they may or may not have with gliomagenesis.

Nestin

Nestin is a filament marker of immature neurons and stem cells expressed during development. It has been proposed that nestin expression in gliomas is related to improved cell motility, invasive potential, dedifferentiated status, and increased malignancy. It has been shown that 46 % of primary CNS tumors are nestin expressing and this expression level increases with increased malignancy and grade in astrocytomas [50]. Other groups have found correlation in HGGs with respect to nestin expression [51, 52]. Clinical expression of stem cell markers has been correlated with survival and prognosis in many different grades of gliomas; however, it has rarely been shown to correlate to tumorigenicity in vitro or in vivo.

Integrin α6

Integrin α6 is a component of the extracellular matrix and its contact is important for glioma stem cell's maintenance and the interaction with laminin-expressing endothelial cells. The targeting integrin α6 in GBM cells results in inhibition of proliferation, tumor formation, and the ability to self-renew. In the normal adult brain, integrin α6 has shown to regular neural stem cell growth and play and important role in the SVZ of the lateral ventricles [53]. Glioblastoma patient biopsies revealed that integrin α6+ cells localize in close proximity to the tumor vasculature and often coexpress the stem cell markers CD133 and nestin. Integrin α6 has also shown to be capable of enriching cells in vitro with or without CD133 for high self-renewal capacity. In addition, lentiviral targeting of integrin α6 with shRNA reduced both sphere forming ability and tumorigenic potential. As well, in vivo transplantation assays with integrin α6+ cells resulted in high incidence of primary and secondary tumor formation with a correlation to reduced survival when compared to integrin α6− cells. Integrin α6 has shown to be another potential target for antiglioma therapy.

Musashi-1

Musashi-1 is an RNA-binding protein and belongs to a family of evolutionarily conserved neural RNA-binding proteins. Musashi-1 expression in tumor cells has shown to be a positive marker for gliomasphere formation, as well as its ability to

self-renew and differentiate into different cell types. Specifically in astrocytomas, high musashi-1 RNA expression has correlated to increased tumor grade. However, no correlation currently exists for clinical survival or prognosis factors [8].

EphA2 and EphA3

Eph receptors are the largest family of receptor tyrosine kinases and have vital cell functions, such as cell adhesion, migration, and axon guidance during development. Eph receptors and Ephrin ligands are expressed at their highest during embryonic development and evidence suggests a role in the regulation of stem cell differentiation and cellular fate. The expression levels of Ephrins and Eph receptors are known to be aberrant in HGG, such as glioblastoma. Deregulation of the Eph receptor/ephrin system is associated with tumorigenic properties, tumor growth, angiogenesis, and metastasis through epithelial-to-mesenchymal transition (EMT). EphA2 receptors are overexpressed in epithelial malignancies and glioblastoma, where it promotes proliferation and invasion. Binda et al. showed that in glioblastoma, expression levels of EphA2 vary within a tumor population and is representative of the tumor-initiating population of cancer cells [54]. EphA3 receptors are overexpressed in leukemia, lymphoma, lung cancers, melanoma, and gastric carcinomas. Day et al. also showed EphA3 to be highly expressed in the tumor-initiating population of cancer cells and is critically involved in tumorigenic potential [55]. Somatic mutations in EphA3 have been identified in glioblastoma, making the two potential candidates for stem cell markers. Due to the lack of expression within normal human tissue and the experimental evidence that suggests these two markers are indicative of the stem-like cells within GBM, they may be future therapeutic targets for glioblastoma.

Embryonic Stem Cell Markers

There are a large number of stem cell markers that have been identified during neurogenesis of the fetal brain. Markers such as Oct4, Sox2, and Nanog have all been extensively researched in neural stem cell development. Sox2 is necessary for normal pluripotent cell development and maintenance during neurogenesis. The loss of Sox2 in embryogenesis is correlated with a loss of pluripotency and self-renewal. The knockdown of Sox2 in glioma results in diminished tumorigenesis. However, clinically it has been shown not to be a prognostic factor. Oct4 is also expressed in pluripotent embryonic stem and germ cells. It also regulates self-renewal and differentiation during neurogenesis. It has found to be expressed in a variety of cancer types and when knocked down has shown to enhance sensitivity to chemo- and radiotherapies whereby increasing apoptosis [56]. Nanog is a transcription factor that is also responsible for pluripotency and stem cell self-renewal maintenance

during embryogenesis. Clinical expression of nanog has not been successfully linked to HGG; however, its activation and mutagenic capabilities have been explored as a possible etiology of stem cell activation by MET-proto oncogenes [57]. Further investigation is needed to fully understand how these embryonic stem cell factors play a role in gliomagenesis.

Therapeutic and Prognostic Implications from Stem Cell Markers

The ability to identify and quantify a specific stem cell marker in patient tumor biopsies could provide a much better prognostic and therapeutic tool for clinicians to diagnose and treat tumors of the CNS. However, the irreproducibility and the subsequent large body of conflicting results make it difficult to say whether or not the specific stem cell markers can be used to identify and isolate glioma stem cells. As the stem cell population represents the potential pool of tumor cells resistant to treatment and capable of highly malignant regrowth, many different markers can be expressed on CSCs that are capable of producing tumor populations and recapitulating primary patient tumors [58]. The main problem is the oscillation of marker expression in a cell-cycle dependent manner. This causes an emerging complication for therapeutic-suitable markers as the oscillation between quiescent and activated states results in cells that can undergo reversion back and forth between differentiated and progenitor states. The current use and practice of classifying cell surface proteins in an attempt to identify CSCs have shown themselves to be insufficient in presenting stem-cell-like properties of glioma cells.

CSC Radio and Chemotherapy Resistance

With the influx of multiple potential stem cell markers numerous research groups have attempted to understand the difference in these cell types compared to the bulk tumor mass. CD133 is currently the most studied stem cell marker for HGG, attempting to comprehend the high rates of patient resistance to conventional treatments and the low 5-year survival statistic. Within the research on CD133+ cells it has been shown that this population to be more chemoresistant and radioresistant to conventional therapies. There are potentially multiple mechanisms that may be responsible for this resistance. First, it has been shown that the initiation of autophagy can play a role in glioma stem cell radioresistance [59]. Cells treated with ionizing radiation (IR) induced autophagy in CD133+ cells as opposed to CD133− cells, and therefore expressed more autophagy-related proteins. Autophagy within cancer cells allows for the evasion of apoptotic mechanisms as well as provides substantial proliferative nutrients. When silenced with an autophagy inhibitor, the CD133+

cells became sensitized to the IR and resulted in a significant reduction in survival and the ability to form neurospheres in culture. Another possible mechanism of resistance is the inflammatory mechanism from Cox-2. There is evidence that Cox-2 expression in CD133+ glioma stem cells has a direct effect on NFkB mediated Cox-2 regulation, resulting in increased survival in a toxic environment [60]. With this in mind the potential for Cox-2 inhibitors may result in radiosensitization of high-grade glioma stem cells. Furthermore, glioma stem cells have shown to be completely resistant to tumor necrosis factor related apoptosis inducing ligand (TRAIL), whereas non-stem cells have a moderate sensitivity. Glioma stem cells seem to express minimal levels of caspase 8 (CASP8), an mRNA, and protein known to be necessary for TRAIL-induced apoptosis. Additionally, glioma stem cells have shown to have a hypermethylated CASP8 promoter, which is not seen in the non-stem glioma cells [61]. Another avenue of resistance has been shown in the ATP-binding cassette subfamily G member 2 (ABC-G2). It was discovered that ABC transporter function was increased in side populations of glioma cells and the expression levels to positively correlate with increased pathological glioma grade in the U87 glioma cell line [62]. The standard of care treatment for glioma patients is chemotherapy with Temozolomide (TMZ), and although this does not activate ABC-G2 substrates, it was found to increase the side populations of glioma stem cells, especially in cells missing the tumor suppressor PTEN. The activation of Tie2 receptors results in the increased expression of ABC transporters and may be clinically relevant in the future evolution of glioma treatment with TMZ. The role of Tie2 has been shown using small interfering RNA (siRNA), whereby the treatment of glioma stem cells with Tie2 siRNA results in ablation of chemoresistance to TMZ [63]. These are just some of the possible evasive mechanisms that glioma stem cells possess. Further research into the complexity that surrounds the tumor microenvironment and the ineffectiveness of current standard treatments will hopefully unfold this multifaceted deadly disease.

Possible Treatment Options to Target Glioma Stem Cells

Following the idea and acceptance of the existence of glioma stem cells came the notion of potential treatment options to target this population of cells. The possibility of directly targeting the glioma stem cell itself or targeting the tumor microenvironment that houses all of the extensive evasive mechanisms has been proposed. Direct targeting of glioma stem cell resistance to conventional therapies, blocking its function, and inducing differentiation have all been proposed. Important experiments have demonstrated that during IR glioma stem cells preferentially activate DNA checkpoint kinases (Chk1/Chk2), which results in a greater degree of DNA repair. With the glioma stem cell fraction of cells being potentially higher than non-stem cells in the residual tumor, it is not surprising that post-IR there is a high rate of recurrent gliomas. The possibility of checkpoint blockages that could induce glioma stem cell radiosensitization may cause a reduction in the number of

surviving fractions post-IR. Along these lines, the primary DNA-based repair mechanism post-TMZ is the overexpression of O6-methylguanine-DNA methyltransferase (MGMT), an enzyme that reverses DNA alkylating damage. The ability to identify and downregulate MGMT status within glioma stem cells may result in increased sensitivity to conventional chemotherapies. Indirect targeting of glioma stem cell would involve deactivating the immune niches, or the perivascular hypoxic microenvironment, with targets such as VEGF, mTOR, hypoxia inducible factor (HIF), or STAT3 [60].

Many of these concepts have high-reward possibilities in patients with HGGs. However, the current ability to identify and segregate the population of stem cells within a glioma cell population is lacking. Nevertheless, research strides are still being made in understanding the complexity of CNS tumors and will continue to make strides for decades to come. Possible treatment avenues of glioma stem cells have been opened up to patients clinically and the resulting information will only aid in the ability to treat patients of the future. There may never be one defining criteria for a glioma stem cell, let alone a cancer stem cell, and this owns to the ever-changing and highly sophisticated heterogeneity that is cancer.

Bone morphogenic proteins (BMPs) have an instructive role in the adult brain stem cell niche, which elicits multiple actions, and a high favoring of differentiation into astroglial fates. Due to these significant roles in neural stem cells, BMPs have been investigated as to their potential antitumor efficacy on brain tumors. BMP4 has shown to elicit the strongest effects, triggering significant reductions in the stem-like, tumor-initiating precursors of GBMs. Piccirillo et al. specifically showed that BMP4 and its receptor (BMPR) transcripts and proteins are expressed in GBM cells and specifically within the CD133+ population. They also showed that in vivo administration of BMP4 effectively blocks tumor formation and the associated mortality in 100 % of mice intracranially transplanted with human GBM cells. Due to the specificity and the non-cytotoxic therapeutic advantage, BMP4 may be an effective treatment option in patients [64].

Immunotherapy and Stem Cells

With the push for new and innovative treatment options for HGG patients, adjuvant experimental therapies, such as immunotherapy, are being explored. Immunotherapy for glioma stems from the idea that cancer evolution can be attributed to an ability to evade the immune system. In the recent decade, our understanding of the brain and its immune-capabilities in combination with the discovery of tumor-specific T-cell recognizing antigens has brought about the idea of immunotherapy in brain cancer. Currently, there are two types of immunotherapy being explored for glioma patients: active and passive. Active immunotherapy is defined as immunization of patients upon activation of endogenous immune cells. The most common approach is the utilization of autologous dendritic cells (DC) to activate the patient's immune system, whereby passive immunotherapy is defined as immunization of patients

upon the adoptive transfer of ex vivo activated cytotoxic effector cells [65]. Effector cells can be administered systemically or intracranially directly within the tumor. Intratumoral injection of effector cells may be autologous or allogeneic to the patient. Specifically, cytotoxic T lymphocytes (CTL) that are sensitized to glioma-associated antigens and exhibit human leukocyte antigen (HLA) restrictions are commonly used. Clinical trials exploring active immunotherapy currently outnumber passive trials and investigators are testing various immune cell approaches on patients before they exhibit tumor recurrence. Upon tumor recurrence tumors are classically more difficult to treat. The most promising active immunotherapy approach for recurrent GBM comes from Mitchel et al. and their autologous DC approach. Patients are treated with autologous DCs that are pulsed with mRNA isolated from their own CD133+ tumor stem cells. This group is currently moving into phase II clinical trials, but other groups have shown this to be an effective method of immunotherapy in mouse glioma models [66].

Gene Therapy and Stem Cells

In contrast to immunotherapy, gene therapy has been proposed as a possible treatment option to cure patients by treating the underlying cause of the disease. In cancer, this can be a daunting task as patient-specific genetic mutations can number in the hundreds and be mutually exclusive from one another. In gene therapy, viral vectors such as retroviruses, adenoviruses, and adeno-associated viruses (AAV) are commonly used depending on the particular disease type. Each of these viral vectors has limitations: retroviruses need cells to undergo division, AAVs have no efficient production method, and adenoviruses have the potential to trigger immune responses. Nonviral vectors are also available, such as liposomes, and have proven to be noninfectious and non-immunogenic [67]. These are, therefore, safer for the patient, but have limited antitumor efficacy. Due to the high mutation rate and the impracticality of sequencing entire patient genomes, targeting patient resistance to conventional treatments is a novel approach to gene therapy in glioma. Approximately, 50 % of HGG patients overexpress methylguanine methyltransferase (MGMT), indicated by hypomethylation of the MGMT promoter, controlling its expression. As discussed, high MGMT expression levels clinically results in resistance to current chemotherapy approaches with TMZ. This resistance is due to the capability of the catalytic MGMT protein to repair TMZ-induced cytotoxic DNA damage, which confers with poor patient survival (median survival = 12.6 months). To combat this resistance the use of a small nucleoside inhibitor, O^6-benzylguanine (O^6BG), has shown to effectively deplete MGMT activity. O^6BG mimics the methylation of the guanine nucleotide, which is the base targeted by MGMT, whereby it binds to the MGMT protein and instigates structural changes that denote the protein–nucleotide complex for degradation. Treating patients with O^6BG has shown to reestablish tumor cell sensitivity to alkylating agents, such as TMZ. However, during phase I studies it was shown that

combination of O^6BG/TMZ treatment resulted in severe off-target myelosuppression, whereby patients exhibited high incidence of neutropenia. This hematopoietic-specific toxicity was attributed to low-to-nonexistent levels of MGMT within the hematopoietic stem cells (HSC) and progenitor cells. To combat this toxicity, Adair et al. [68] conducted a clinical trial utilizing gene therapy to determine if O^6BG resistance in HSCs improves chemotherapy tolerance and outcome. Adair's group has previously shown that the expression of O^6BG-resistant MGMT mutant P140K by HSC provides significant chemoprotection against hematopoietic toxicity following O^6BG/TMZ therapy. This group has initiated a phase I/II clinical trial that included newly diagnosed $MGMT^{hi}$ GBM patients to test the chemoprotection. Patients accepted were transplanted with autologous P140K gene-modified hematopoietic $CD34^+$ cells to attempt to prevent the associated HSC toxicity seen during the combinatorial O^6BG/TMZ chemotherapy. To accomplish this, they genetically modified enriched patient $CD34^+$ cells with a retroviral vector encoding the P140K transgene. To date, seven patients have been treated with this approach and all reported increased chemotherapy tolerance and improved clinical outcomes for all patients. This avenue of treatment is a novel use of patient HSCs in combating chemotherapy-associated toxicity, which can limit the effectiveness. Further studies into stem cells and chemotherapy could greatly benefit brain tumor patients.

Final Summary

In conclusion, this body of work encompasses the high points of current cancer stem cell research and knowledge, with a specific emphasis on glioma stem cells. Glioma stem cells have emerged as an exciting avenue for potential cancer treatments as it is becoming widely accepted that this population of tumor cells is resistant to treatment and result in a sustained tumorigenic population. The ability to identify and target these cells for cytotoxic therapy is still under intense investigation and scrutiny. Furthermore, the debate as to etiology of primary brain neoplasms is still unclear. The diverse cellular heterogeneity and the ability to evade standard treatments by changing tumor cellularity make it very difficult to unravel the sequence of events that leads to a brain tumor. Driver mutations of oncogenesis causing aberrant cellular transformation lead to further mutagenesis making it ambiguous as to where the cancer stem cell lies in the hierarchy of tumor formation. Theories have been postulated and effectively elaborated in this chapter; however, it is important to note that there may be immense areas of theory overlap in the actual patient etiology of cancer. No two patients have the exact same genotypic tumor profile, yet clinical correlations can be made between patients with similar phenotype and pathology. The evolution of the cancer stem cell may be different from one patient to another depending on the timeline of inherent mutations and cellular divisions that give rise to more or less cancer stem cells. Nevertheless, there is a distinguishable population of cells within a malignant growth that are responsible for the

continuous unrelenting tumorigenic growth of cancer. The ability to target this cellular population may be the key to unlocking a viable treatment option for patients with tumors of the CNS.

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Part III
Novel Pharmacological Approaches

Chapter 19
Discovery of Potent Gamma Secretase Modulators for the Treatment of Alzheimer's Disease

Kevin D. Rynearson, Rudolph E. Tanzi, and Steven L. Wagner

Abstract Substantial progress in our understanding of the pathogenesis of Alzheimer's disease (AD) has occurred over the past 25 years. However, we are still in search of a truly disease-modifying therapeutic agent capable of either preventing or significantly delaying the progression of this fatal neurodegenerative disorder. In this chapter, we describe the discovery methods, optimization, target deconvolution, and preclinical development of a novel series of compounds known as gamma-secretase modulators or GSMs. We also describe the rationale behind why we believe GSMs are ideally suited as a therapeutic approach for the heritable early-onset form of AD (EOAD). Importantly, we contrast GSMs from gamma-secretase inhibitors (GSIs) from the standpoint of side effects and mechanism of action. Furthermore, we differentiate the two major classes of GSMs: (1) Carboxylic acid-containing NSAID derived GSMs and (2) non-NSAID derived GSMs from a structural, mechanistic, and pharmacological perspective. Finally, we will describe the types of clinical trials that we feel hold the most promise for the demonstration of disease-modifying efficacy for these types of GSMs.

Keywords Gamma-secretase modulators • GSM • Alzheimer's disease • Therapeutic • Disease-modifying treatment prevention

Alzheimer's disease (AD) is an enormous problem for our healthcare system and may become an even greater health and economic burden as the baby boomers reach the age most commonly affected (>65 years) by the disease [1]. AD is the most common cause of dementia and currently only palliative treatments exist that

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provide only a modest and temporary symptomatic benefit with no effect on disease progression [2]. AD is characterized pathologically by an abundance of neuritic plaques and neurofibrillary tangles (NFTs) in brain regions important for cognition [3]. Neuritic plaques are composed primarily of the Aβ42 peptide variant [4], and an increased ratio of Aβ42/Aβ40 represents the most consistent biochemical phenotype of more than 200 different familial AD or FAD-linked mutations [5]. In fact, over 25 years of published studies, including those providing genetic, biochemical, pathological, and epidemiological evidence, lend substantial support to the theory that alterations in the relative levels of the Aβ42 and Aβ40 peptide species, i.e., Aβ42/40 ratio, play a pivotal role in the pathogenesis of AD [3]. NFTs are composed of paired helical filaments containing hyperphosphorylated aggregates of the protein tau. The lesions characterized as neuritic plaques and NFTs represent the critical pathological components of AD; however, this chapter will focus on a therapeutic approach exclusively aimed at diminishing the levels of neuritic plaques which are composed predominantly of Aβ peptides, especially Aβ42.

Generation of Aβ peptides requires sequential proteolysis of the amyloid precursor protein (APP) by two distinct enzymes. β-site APP cleaving enzyme (BACE 1 or β-secretase) is responsible for the first cleavage event which generates the carboxy-terminal APP BACE 1 product known as APP-β-CTF [6]. Then proteolysis of the APP-β-CTF product by γ-secretase generates the various Aβ peptides ranging in length from approximately 34–43 amino acids [7]. Consequentially, one of the initial approaches for the therapeutic intervention of AD focused on lowering the amount of total Aβ peptide production by inhibiting the catalytic activities of either BACE-1 or γ-secretase.

γ-Secretase is a heterogeneous membrane enzyme [7] composed of four individual proteins: presenilin, anterior pharynx-defective 1 (APH-1), nicastrin, and presenilin enhancer 2 (PEN-2) as shown in Fig. 19.1. γ-Secretase regulates intramembrane proteolysis of APP [8] in addition to a rather large number of other type I membrane protein substrates, including the Notch-1 receptor [9]. γ-Secretase-mediated cleavage of the Notch-1 receptor at site 3 (S3), also known as the epsilon-site or ε-site, yields a large cytoplasmic peptide referred to as the Notch intracellular domain (NICD). Following formation, the NICD then translocates to the nucleus and alters the expression of genes necessary for proper cellular differentiation and the development of key organs [10]. γ-Secretase inhibitors (GSIs) prevent proteolytic processing at both the ε-sites and γ-sites of the enzyme's numerous substrates including APP (see Fig. 19.2) and the Notch-1 receptor, resulting in a number of adverse effects secondary to inhibition of ε-site cleavage of the Notch-1 receptor and APP, thereby preventing NICD and AICD formation, respectively [11, 12]. GSIs also cause accumulation of APP-β-CTFs which cause cholinergic degeneration in mouse models of Down Syndrome [13] and AD [14]. Eli Lilly and Co recently reported (http://www.lilly.com) during phase 3 clinical trials of semagacestat, a GSI, that treatment led to worsened cognition and worsened performance of activities of daily living in mildly to moderately affected AD patients. Similar results were also reported for another GSI, avagacestat or BMS-708163, an arylsulfonamide-containing compound that is quite structurally distinct from semagacestat following

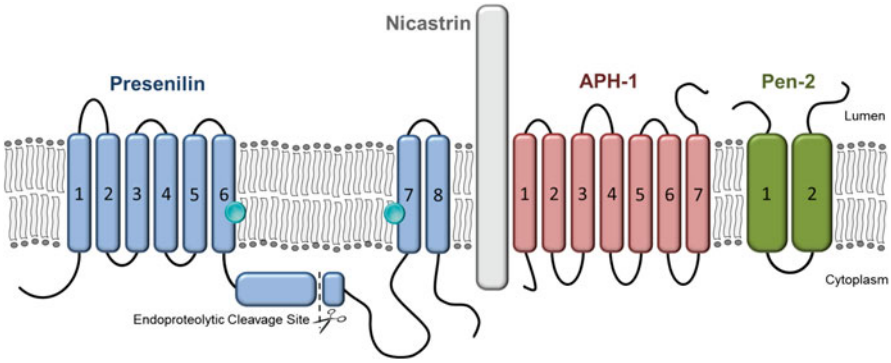

Fig. 19.1 Schematic depiction of the γ-secretase enzyme complex. The four critical components of the γ-secretase enzyme complex: presenilin, nicastrin, anterior pharnyx defective 1 (APH-1), and presenilin enhancer-2 (Pen-2). Full length presenilin is endoproteolyzed into an amino-terminal fragment (PS-NTF) and a carboxyl-terminal fragment (PS-CTF). The two aspartic acid residues comprising the active site of the γ-secretase enzyme complex are designated by blue circles on the PS-NTF and the PS-CTF

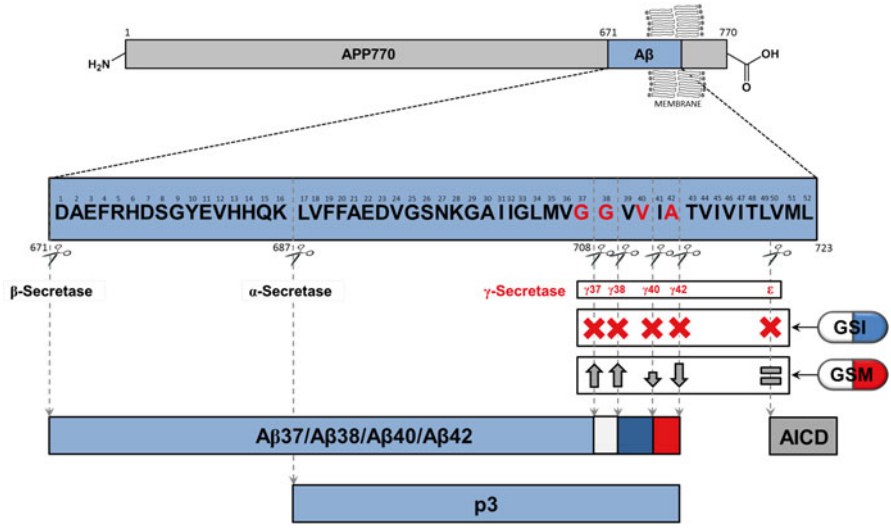

Fig. 19.2 Schematic diagram depicting various proteolytic cleavages of the 770 amino acid isoform of APP by β-secretase, α-secretase, and γ-secretase. The γ-secretase ε-site cleavage between L49 and V50 generates the AICD and the γ37-, γ38-, γ40-, and γ42-site cleavages generate Aβ37, Aβ8, Aβ40, and Aβ42 peptides, respectively. The p3 peptides are the products of the α-secretase cleavage and the γ-site cleavages. The GSI blocks both the ε cleavage and the γ-site cleavages. The GSM has no effect on ε cleavage, inhibits the γ42-site cleavage and to a lesser degree the γ40-site cleavage and potentiates both the γ38-site and the γ37-site cleavages

a phase 2 trial in mildly to moderately affected AD patients (http://www.bms.com). Aside from efficacy issues, both GSIs demonstrated severe side effects, thus indicating a serious liability associated with this therapeutic strategy.

In view of the severe consequences associated with inhibiting both the ε-site and γ-site cleavages of the γ-secretase complex, which has been proposed to function as a "proteasome" for type I membrane proteins [15], a more prudent therapeutic approach was to identify small molecules that could preferentially lower the levels of the most fibrillogenic Aβ peptide, Aβ42, without affecting γ-secretase catalytic activity at the ε cleavage sites. This type of noninhibitory strategy has been validated in both cell culture models and AD animal models using nonsteroidal anti-inflammatory drug (NSAID)-like compounds to achieve modulation of γ-secretase activity and attenuate the Aβ42/Aβ40 ratio without affecting ε-site proteolysis [16, 17]. This conceivably safer and more selective approach for reducing Aβ42 generation initially utilized NSAID-like substrate-targeted GSMs (e.g., tarenflurbil, also referred to as R-flurbiprofen or flurizan) which have been shown to selectively inhibit Aβ42 formation and augment the production of the shorter Aβ38 peptide variant; however, the poor potency of this class of compounds, combined with their limited ability to cross the blood–brain barrier, resulted in a lack of efficacy in the clinic [18].

The appeal of this therapeutic rationale based on the apparent mechanism of action of these first-generation NSAID-like GSMs warranted a much more thorough drug discovery-based search for truly potent and bioavailable small molecules capable of selectively lowering Aβ42 levels in the brain while preserving the endogenous catalytic activity of γ-secretase toward critical ε-site cleavages of the Notch-1 receptor, APP, and other important γ-secretase substrates.

The typical approach for an empirical drug discovery effort (see Fig. 19.3) encompasses selecting the target or perhaps the target hypothesis, which in this case was modulating γ-secretase, since the goal was to identify small molecules capable of selectively reducing the generation of Aβ42 and without affecting either NICD or AICD generation from ε-site proteolysis of the Notch-1 receptor and APP, respectively. A phenotypic-like cell-based assay was employed as a primary screening assay where the production of two key Aβ peptide variants, Aβ42 and Aβ40, was measured in media from cells stably overexpressing wild-type

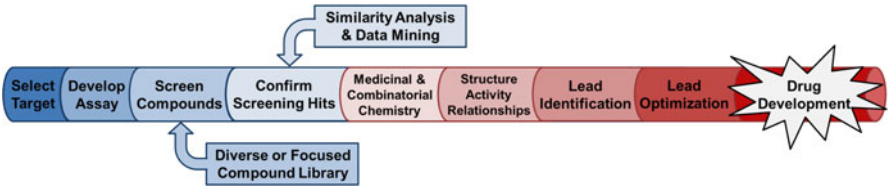

Fig. 19.3 Schematic representation of the various stages of small molecule drug discovery and development

human APP [19]. A diverse compound library was then selected using various computational chemistry approaches in order to assemble a chemically divergent set of approximately 80,000 small molecules with drug-like properties [20] for high-throughput screening (HTS). The 80,000 compound library was purchased from a variety of commercial sources and formatted into 384-well plates. The highly sensitive primary screening assay was then adapted into a miniaturized high-throughput immunochemical assay capable of measuring levels of Aβ42 and Aβ40 (Aβ42/Aβ40 ratios) in media from a cell line stably overexpressing human wild-type APP in a 384-well format [19]. Using a monoclonal antibody-based homogeneous fluorescence resonance energy transfer (FRET) HTS assay, a compound which had an IC_{50} value of 15 μM for the inhibition of Aβ42 production was discovered. In order for the hit compound to be validated, the ligand was first resynthesized, and the structure was spectroscopically confirmed by NMR and mass spectrometry. The resynthesized ligand's activity was confirmed by reevaluation in the primary screening assay and in a number of additional assays which include concentration response curve and secondary and orthogonal assays. These assays included measuring effects on additional Aβ peptide variants such as Aβtotal, Aβ40, and Aβ38, as well as orthogonal assays, which required that the compound performed appropriately in a completely different immunochemical-based assay format using a different set of monoclonal antibodies against the same biochemical marker used for the primary HTS assay (Aβ42). Since our confirmed hit passed all subsequent screening criteria of the first two levels of the testing funnel depicted in Fig. 19.4, several focused chemical libraries were then designed and synthesized using high-throughput organic synthesis (HTOS) and combinatorial chemistry techniques based on the structure of this confirmed hit, along with computational structural similarity analyses and additional data mining.

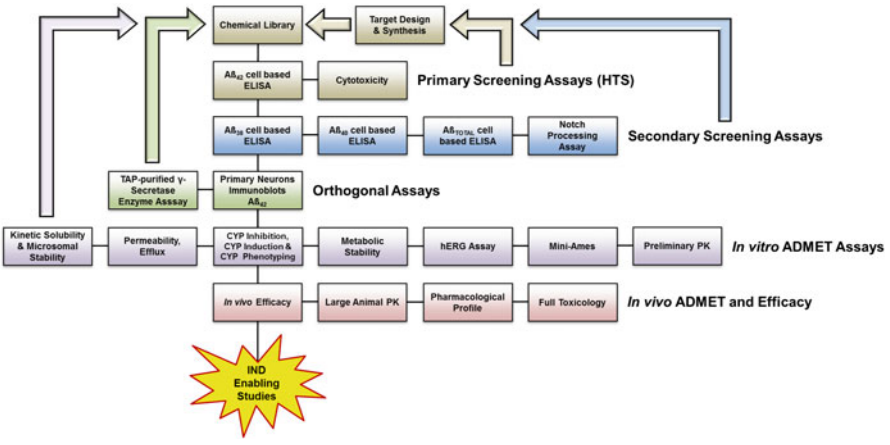

Fig. 19.4 Testing funnel depicting the various types of assays utilized for the screening, optimization and development of GSMs

Proceeding along the horizontal scheme of Fig. 19.3, we ultimately synthesized over 1200 analogs performing both lead optimization and lead evolution medicinal chemistry efforts and evaluated structure–activity relationships (SAR) over a period of 3–4 years. Following this considerable effort in medicinal chemistry, a large fraction of these compounds had IC_{50}s for inhibiting Aβ42 production in the mid-nM range ($IC_{50} \leq 200$ nM) with several promising compounds exhibiting IC_{50}s for inhibiting Aβ42 production in the single digit nM range (<10 nM) [19].

Prior to reaching the lead optimization phase of this discovery effort, the lead compound had to pass a number of additional tests beyond simply showing excellent potency in the primary screening assay. We then subjected the best compounds to the entire testing scheme or testing funnel depicted in Fig. 19.4. This included, in addition to the primary, secondary, and orthogonal assays, a number of in vitro absorption, distribution, metabolism, excretion, and toxicity (ADMET) assays. These in vitro ADMET studies are used to eliminate scaffolds that are likely to perform poorly in subsequent in vivo pharmacokinetic/pharmacodynamics (PK/PD) studies and to prioritize compounds for these expensive animal studies. Further SAR studies identified compounds suitable for focused lead optimization medicinal chemistry studies. Compounds with the best overall profiles were subjected to preliminary PK analyses which contain an oral (p.o.) and an intravenous (i.v.) administration arm. These in vivo assays informed us of the extent to which these molecules penetrate the brain. Compounds having the best brain/plasma ratios and best overall PK parameters which include the compound's half-life ($t_{1/2}$), time of maximal exposure (T_{max}), concentration at maximal exposure (C_{max}), clearance (Cl), and % oral bioavailability (F) were selected for in vivo studies in a transgenic animal model of AD, the Tg2576 transgenic mouse model [21].

In parallel with these in vitro and in vivo ADMET studies, target deconvolution studies were carried out that entailed immobilizing a potent GSM onto an agarose matrix and performing affinity chromatography of cellular extracts. Pen-2 and the PS-1-NTF were shown to bind to the immobilized GSM ligand. In addition, when tandem affinity purification (TAP) was used to isolate the intact γ-secretase enzyme complex, GSM *4* was able to bind and modulate the highly purified γ-secretase enzyme complex in a reconstituted enzymatic assay [19, 22]. Interestingly, other GSMs within this imidazole-based subclass have unequivocally been shown to bind to the γ-secretase enzyme and not the APP substrate. Studies by several independent groups utilizing photoaffinity cross-linking probes have all demonstrated binding of this subclass of GSMs to the PS-1-NTF [23].

The overall best compound optimized by our team was the diarylaminothiazole depicted in Fig. 19.5 as compound *4*. In terms of in vitro potency, this compound was over 1000-fold more potent than the NSAID-like carboxylic acid GSMs ibuprofen and tarenflurbil [19]. In acute efficacy studies using female Tg2576 transgenic mice, administration of compound *4* at doses of 25 mg/kg p.o. and 50 mg/kg p.o. reduced Aβ42 levels in brain by ~15 % and 30 %, respectively [19]. At the higher oral dose of 100 mg/kg, compound *4* reduced the brain levels of Aβ42 by approximately 40–50 % [19].

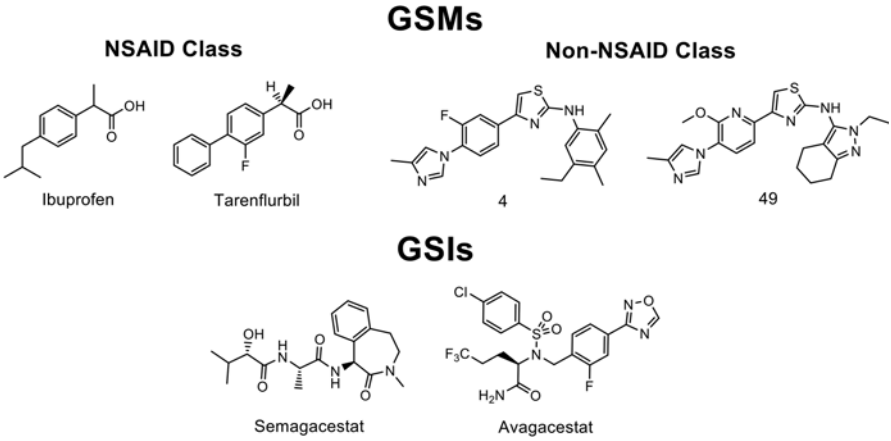

Fig. 19.5 Chemical structures of GSMs from the NSAID class and the non-NSAID

Chronic efficacy studies with compound *4* were carried out to assess effects on neuritic plaque formation in the Tg2576 transgenic mouse model. When 8-month-old pre-plaque bearing Tg2576 female transgenic mice were exposed daily to approximately 50 mg/kg of compound *4* for 7 consecutive months, there was a dramatic reduction in the number of neuritic plaques in both the hippocampus and throughout the cerebral cortex compared to mice fed normal chow [19]. The compound was also very well tolerated and showed no effects on weight gain and most importantly showed no side effects such as goblet cell hyperplasia or skin color changes which are reproducibly seen upon repeated exposure of GSIs to rodents and transgenic mice [24–26].

A potential concern with developing this type of GSM (e.g., compound *4*, an aminothiazole-bridged aromate) is the inherent lipophilicity. Highly lipophilic compounds have a greater tendency of having off-target toxicities and have proven to be more difficult to develop, primarily because of their poor aqueous solubilities [27]. Safety and toxicity studies that are required for filing an investigational new drug (IND) application for clinical testing require supra-efficacious dosing regimens that demand relatively high concentrations of drug. These concentrations are often difficult to achieve for lipophilic compounds unless extensive formulation work is performed.

In order to circumvent this potential liability, we identified permissive aryl moieties in compounds such as compound *4* (a diarylaminothiazole) and substituted with specific heterocycles (see compound *49* in Fig. 19.5). Replacement of the aryl groups with certain heterocycles led to dramatically improved physicochemical properties including increased kinetic and thermodynamic solubilities in aqueous buffers [22]. These more soluble GSMs or SGSMs were also shown to be just as potent as the more lipophilic series and appear to be much more suitable for IND-enabling safety and toxicity studies [22].

Provided these SGSMs are successful in attaining an IND from the Food and Drug Administration (FDA), they should initially be tested for efficacy in selected patient populations such as early-onset familial AD (EOFAD) patients. These patients harbor dominantly inherited mutations in either PS1, PS2, or APP and the vast majority of these carriers display the biochemical phenotype of an increased Aβ42/Aβ40 ratio [28]. This is due to a significant increase in the absolute Aβ42 production rate and in the Aβ42/Aβ40 production rate ratio in mutation carriers versus nonmutation carriers [29]. These patients, although accounting for only 1 % of AD, would be ideal for a primary prevention clinical study to test the effects of SGSMs on disease prevention or on disease progression because the age at clinical onset is very similar from generation to generation as is the duration of illness which depends primarily on the particular mutation and background family genetics [30].

We established a target product profile for the "ideal" and "acceptable" SGSM (Table 19.1), which describes minimally acceptable as well as ideal results for various pharmaceutical properties such as the therapeutic indication, the patient population, the mode of delivery, the duration of treatment, the dosing regimen, and the level of efficacy. Perhaps, primary prevention trials utilizing EOFAD patients would be the ideal patient population in which to launch these initial clinical trials once these compounds are proven safe in humans. Hopefully, this will occur within the next year or two (2015–2016) if not sooner.

Table 19.1 Target product profile for a gamma-secretase modulator

Drug properties	Minimum acceptable result	Ideal result
Primary drug indication	Prevention or statistically significant reduction in rate of cognitive decline/year (ADAS-cog)	Prevention or statistically significant reduction in rate and extent of cognitive decline/year (ADAS-cog)
Patient population	• FAD mutation carriers (1° prevention) • Presymptomatic PET positive (2° prevention) • Adults with mild cognitive impairment (MCI) and mildly affected Alzheimer's disease (AD) patients	• FAD mutation carriers (1° prevention) • Presymptomatic PET positive (2° prevention) • Adults with MCI and mildly to moderately affected AD patients
Delivery mode	Oral	Oral
Treatment duration	Chronic	Chronic
Regimen	2×/day	1×/day
Efficacy	Prevention or a ≥25 % reduction in rate of cognitive decline/year in FAD mutation carriers, presymptomatic PET positive, MCI, and mildly affected AD patients	Prevention or a ≥25 % reduction in rate of cognitive decline/year in FAD mutation carriers, presymptomatic PET positive, MCI, mildly and moderately affected AD patients

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Chapter 20
Blocking the Nogo-A Signaling Pathway to Promote Regeneration and Plasticity After Spinal Cord Injury and Stroke

Anna Magdalena Guzik-Kornacka, Flóra Vajda, and Martin E. Schwab

Abstract Myelin of the central nervous system (CNS) exerts an inhibitory effect on growing neurites, leading to restricted axonal regeneration, limited compensatory sprouting, and permanent functional deficit after CNS injury. Nogo-A is a membrane protein enriched in CNS myelin that plays a key role in neurite growth inhibition. In various animal models, application of function-blocking anti-Nogo-A antibodies and of other Nogo-A signaling blocking agents led to enhanced regeneration and compensatory sprouting after spinal cord injury and increased structural reorganization and plasticity after stroke, both associated with improved functional recovery. This chapter discusses recent advances in understanding how Nogo-A and other glia-derived inhibitors limit regeneration and functional recovery after CNS damage. We summarize the current experimental CNS repair strategies and clinical trials that use reagents which neutralize Nogo-A or suppress Nogo signaling, in particular for spinal cord injury and stroke, with some results also in amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). In the future, pharmacological therapies blocking glia-derived neurite growth inhibitors and promoting neuronal intrinsic growth capacities should be combined with well-timed rehabilitative training to optimize recovery of trauma or neurological patients.

Keywords Myelin • Nogo-A • Nogo-A receptors • Function-blocking antibodies • CNS repair • Axonal regeneration • Plasticity • Spinal cord injury • Stroke • Amyotrophic lateral sclerosis • Multiple sclerosis • Functional recovery • Animal models • Clinical trials

Abbreviations

AIS	American Spinal Injury Association Impairment Scale
ALS	Amyotrophic lateral sclerosis
BBB	Basso, Beattie, and Bresnahan locomotor activity test

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BDA	Biotinylated dextran amine
C	Cervical level
ChABC	Chondroitinase ABC
CNTF	Ciliary neurotrophic factor
CREB	cAMP response element-binding protein
CRMP2	Collapsin response mediator protein 2
CSPG	Chondroitin sulfate proteoglycans
CST	Corticospinal tract
ECM	Extracellular matrix
ER	Endoplasmic reticulum
GPI	Glycosylphosphatidylinositol
ICMS	Intracortical microstimulation
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
i.v.	Intravenous
KLF4	Krüppel-like factor 4
L	Lumbar level
LARG	Leukemia-associated Rho guanine nucleotide exchange factor
LTP	Long-term potentiation
mAb	Monoclonal antibody
MAG	Myelin-associated glycoprotein
MCAo	Middle cerebral artery occlusion
MLC2	Myosin light chain 2
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
NgR1	Nogo-66 receptor 1
NR2d	NMDA-receptor 2d
NT-3	Neurotrophin-3
OMgp	Oligodendrocyte myelin glycoprotein
PNS	Peripheral nervous system
PTEN	Phosphatase and tensin homolog
RHD	Reticulon homology domain
ROCK	Rho-associated protein kinase
RTN	Reticulon
S1PR2	Sphingosine-1-phosphate receptor 2
SCI	Spinal cord injury
SOCS3	Suppressor of cytokine signaling 3
SSH	Slingshot phosphatase
STAT3	Signal transducer and activator of transcription 3
T	Thoracic level
Th cells	T helper lymphocytes
wk	Week

Introduction: Myelin Associated Neurite Growth Inhibitors—Focus on Nogo-A

The Central Nervous System Is a Hostile Environment for Axonal Regeneration: History of Discovery

Already in the early 1900s, Ramón and Cajal described that lesioned axons in the CNS do not regenerate but retract and form dystrophic end-bulbs after an injury; only occasional sprouting over short distances can be observed [1]. This lack of axonal regeneration within the CNS after large lesions leads to irreversible functional deficits, e.g., impaired locomotion or hand function in spinal cord-injured or stroke patients.

In the peripheral nervous system (PNS), a high level of regenerative capacity can enable the reinnervation of target organs, which in turns leads to functional recovery. In 1911, a pupil of Ramón and Cajal, Tello, showed massive ingrowth of silver stained fibers of cortical origin into a peripheral nerve graft implanted into the forebrain cortex of rabbits. Further experiments in the 1980s also demonstrated that PNS axons (that otherwise regenerate) fail to grow in the CNS milieu, but CNS axons elongated for unprecedented distances when the CNS environment was replaced by peripheral nerves [2]. Therefore, these studies demonstrated that under favorable conditions CNS neurons are able to regenerate and opened the avenue for research on determining the molecular differences between the growth permissive PNS and hostile CNS environment.

Several components of the CNS myelin were discovered to inhibit axonal growth after an injury. Experiments in the late 1980s that compared outgrowth inhibition properties of CNS and PNS myelin extracts [3, 4] led to the discovery of Nogo-A (initially named "IN-250") [5, 6], a myelin membrane protein contributing to the regeneration failure in the CNS after an injury [7]. Further components of CNS myelin have been shown to have inhibitory properties: myelin-associated glycoprotein (MAG) [8, 9] and oligodendrocyte myelin glycoprotein (OMgp) [10] are also located in the myelin sheaths of oligodendrocyte and play a role in inhibiting the regrowth of injured neurons, at least in vitro. Versican V2 and brevican, two chondroitin sulfate proteoglycans (CSPG), are present in the CNS myelin and also suppress neurite outgrowth [11, 12]. CSPGs in the extracellular matrix (ECM) contribute to the formation of a growth inhibitory glial scar around the injury site, which together with the aggregation of infiltrating immune and activated CNS glial cells form a barrier for regenerating axons [13]. In addition, sulfatide, a major lipid constituent of CNS myelin, was recently identified as a novel myelin-associated inhibitor of neurite outgrowth [14].

The wide spectrum of myelin inhibitors and ECM molecules that restrict axonal growth raises questions about their physiological function. The onset of myelination correlates with the decline of neuronal growth in the maturing CNS [15], suggesting that myelin and myelin-associated inhibitors might serve as a signal terminating the growth phase leading to the consolidation of neuronal circuits during CNS development [16].

The Neurite Growth Inhibitory Protein Nogo-A: Description of Nogo Signaling and Possible Pharmacological Interventions

Nogo-A (RTN4A) is a 1200 amino acid (aa)-long transmembrane protein containing a C-terminal reticulon homology domain (RHD). Alternative splicing and alternative promoter usage of the *Rtn4* gene give rise to two further proteins, Nogo-B (expressed in many tissue of the body) and Nogo-C (expressed mostly in muscles) [6, 17, 18]. All three Nogo isoforms share the common C-terminus of 180 aas, which consists of two hydrophobic, membrane-anchored regions and a short (60–70 aa-long) hydrophilic region, known as the growth limiting Nogo-66 domain [19]. Nogo-A contains a long 800 aa unique sequence that contains a second neurite growth inhibitory region, the Nogo-A-Δ20 domain (rat aa 544–725) [20].

Nogo-A is enriched in the membrane of the endoplasmic reticulum (ER), where it has been shown to be involved in the formation and maintenance of the tubular ER morphology [21, 22]. However, a functionally active fraction of Nogo-A is localized in the cell surface membrane of oligodendrocytes and neurons [23].

The two highly active inhibitory domains of Nogo-A inducing growth cone collapse and neurite outgrowth inhibition, Nogo-66 and Nogo-A-Δ20, signal through separate receptors, the Nogo-66 receptor1 (NgR1) receptor complex [19] and the sphingosine-1-phosphate receptor 2 (S1PR2) [24], respectively (Fig. 20.1). Both receptors activate the small GTPase RhoA and its effector, Rho-associated protein kinase (ROCK) [25] (Fig. 20.1a).

Multiple ways have been developed to suppress Nogo-A/Nogo receptor interactions and the underlying signaling pathways [26]. Neutralizing Nogo-A either by function-blocking antibodies (IN-1 [7], 11C7 and 7B12 [27], human antihuman Nogo-A antibody (Novartis Pharma, ATI355), humanized anti-Nogo-A antibody (GlaxoSmithKline, GSK1223249, Ozanezumab)) or by interfering with Nogo-A receptors (NEP1-40 [28], NgR1(310)ecto-Fc [29], Lingo-Fc [30], JTE-013) and underlying signaling pathways (pharmacological Rho or ROCK blocker: C3 transferase, Cethrin, Y-27632, fasudil, and KD025) have been shown to reduce the inhibitory effects of CNS myelin on neurite growth, cell spreading, and cell migration in vitro and to boost regeneration, compensatory sprouting, and functional recovery after CNS injury in vivo (Fig. 20.1; red blocking arrows).

Physiological Functions of Nogo-A

In the adult CNS, the bulk of Nogo-A protein is found in myelin [31] and its inhibitory function has been well described for axonal growth, plasticity, and regeneration after CNS injury; reviewed in [32, 33]. The developmental expression of Nogo-A in oligodendrocytes correlates with the course of myelination, and Nogo-A has been proposed to stabilize neuronal circuits at the end of CNS maturation [25]. By controlling spatial segregation and myelin extent, Nogo-A was shown to be a key factor

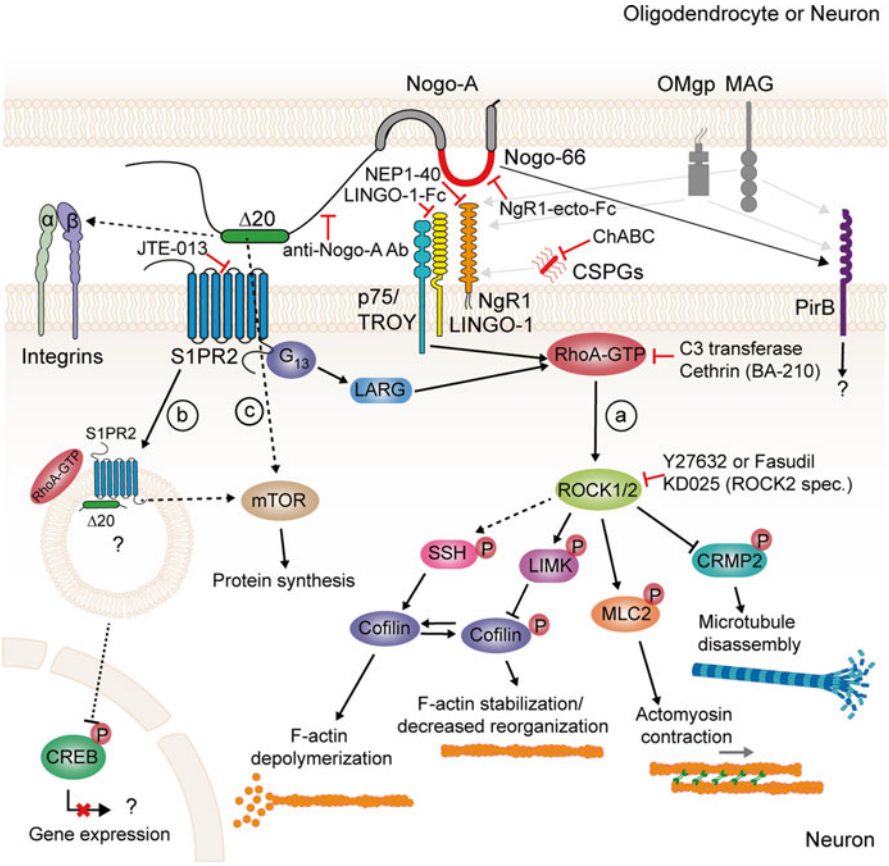

Fig. 20.1 Nogo-A signaling pathways and functional blockers. Nogo-A exerts its inhibitory action mainly through two domains, the Nogo-66 (*red*) and the Nogo-A-Δ20 region (*green*). Nogo-66 binds to the GPI-anchored NgR1 receptor which forms a complex with two co-receptors LINGO-1 and p75 or Troy enabling signal transduction. The NgR1 signaling complex activates RhoA-GTPase which regulates cytoskeleton dynamics and mediates growth inhibitory action of Nogo-A. The Nogo-A Δ20 domain binds to sphingosine 1-phosphate receptor 2 (S1PR2) activating G_{13} protein and further LARG protein which also activates RhoA. (**a**) Therefore, signaling from both Nogo-A inhibitory domains converges on RhoA and its effector, Rho-associated protein kinase (ROCK), leading to F-actin depolymerization, microtubule disassembly and myosin II contractility. (**b**) Nogo-A-Δ20 fragment can be also internalized forming signaling endosomes which modulate growth related gene expression by decreasing CREB phosphorylation. (**c**) The Nogo-A-Δ20 fragment has also been shown to mediate growth cone collapse in a protein synthesis dependent way involving the mTOR pathway. Additionally, the Δ20 region has also been shown to inactivate indirectly integrin signaling. The inhibitors and blocking agents against different molecules involved in Nogo-A, MAG and OMgp and CSPG signaling are depicted on the figure in red: function-blocking antibodies against Nogo-A (IN-1, 11C7, 7B12, human anti-human Nogo-A antibody (Novartis Pharma, ATI355), humanized anti-Nogo-A antibody (GlaxoSmithKline, GSK1223249, Ozanezumab)); NgR1 blocker (NEP1-40); NgR1 or LINGO-1 decoy proteins (NgR1(310)ecto-Fc, LINGO-1-Fc); S1PR2 blocker (JTE-013); CSPG digestion (ChABC); pharmacological Rho or ROCK blockers (C3 transferase, Cethrin (BA-210), Y-27632, fasudil or KD025)

for precise myelination of the developing CNS [34, 35]. Additionally, Nogo-A is also expressed in immature neurons and also in subpopulations of mature neurons [31, 36]. During development, Nogo-A is downregulated in immature neurons at the time of synaptogenesis [37, 38]; however, in plastic brain regions such as the hippocampus, Nogo-A remains expressed and is located at synapses [36], where it was shown to negatively regulate synaptic plasticity [39]. Blockade of Nogo-A, NgR1, or S1PR2 was shown to enhance synaptic plasticity, e.g., long-term potentiation (LTP) in the intact hippocampus and sensorimotor cortex [24, 40–44]. After injury, the effects of neuronal Nogo-A are not yet well understood.

Regeneration and Plasticity after CNS Injury and in CNS Disease

Depending on the lesion size and localization, the consequences of CNS injuries are complex. Patients and experimental animals with large lesions have to cope with permanent and severe functional deficits. Smaller lesions of the spinal cord or the motor cortex have a relatively good prognosis due to adaptive changes in the neuronal circuitries of the spinal cord and brain allowing spontaneous functional improvements [45, 46]. In animal models, injured and spared fibers can sprout and grow collaterals, initiating the remodeling of intraspinal circuits, e.g., by forming "detour" pathways [45, 47]. The distances covered by sprouting fibers are short in general, and the growth response of the neurons is downregulated within a few days, possibly because of the presence of growth inhibitory factors like Nogo-A, CSPGs, or scar-associated molecules [48].

Two key questions and aims in the field of CNS repair are, therefore, an enhancement of the growth capacity of the neurons and overcoming the inhibitory properties of the CNS environment to enable regeneration of fibers over long distances and around large spinal cord or brain lesion sites.

Stimulation of Regeneration by Upregulating Intrinsic Neuronal Growth Mechanisms

Immature CNS neurons in early developmental stages possess robust axon growth and regenerative ability, which is downregulated at adult stages in most types of CNS neurons, possibly through a decrease in trophic support and the influence of inhibition of growth [16, 49, 50]. One way neurite growth and regeneration can be stimulated is the upregulation of the intrinsic growth machinery of neurons, in particular via stimulating the mTOR (mammalian target of rapamycin) and STAT3 (signal transducer and activator of transcription 3) pathways [51]. Eliminating the gene encoding for the tumor suppressor phosphatase and tensin homolog (PTEN)

promoted robust extension of retinal ganglion cell axons and injured corticospinal tract fibers, an effect that was dependent on the mTOR pathway [52, 53]. Although the manipulation of these intracellular growth regulators induces powerful long-distance axonal regeneration in the optic nerve and to a lesser extent in the spinal cord after injury, only few reports on the functional consequences of this neurite growth stimulation exist so far [54]. However, excessive stimulation of mTOR can lead to epileptic seizures and tumor formation, suggesting that over-stimulated neurite growth may lead to the formation of aberrant neuronal connections or that the growth machinery might get out of control and this would result in the overproliferation of glial cells [55, 56]. The downregulation or the deletion of the growth repressor Krüppel-like factor 4 (KLF4), a zinc-finger transcription factor, also increased the number and length of regenerating retinal ganglion cell axons after optic nerve crush [57]. Upregulation of the growth-activating neurotrophic factors such as ciliary neurotrophic factor (CNTF) leads to the activation of the JAK/STAT3 pathway and thereby increased axonal regeneration [58–60]. Upregulation of STAT3 or downregulation of suppressor of cytokine signaling 3 (SOCS3), the negative feedback controller of this pathway, elicited increased axonal regeneration in the lesioned rodent optic nerve [61, 62].

Two extracellular growth inhibitory factors, MAG and Nogo-A, were recently shown to negatively affect the mTOR pathway suggesting that the final growth response is a result of interconnected intrinsic and extrinsic signaling pathways (Fig. 20.1c) [63, 64].

An important consideration for stimulation of axonal growth in the injured adult CNS is that these fibers have to find functionally meaningful targets where they should form synapses, avoiding synapse formation on targets that would lead to malfunctions. Thus, in addition to a growth permissive extracellular environment, guidance and positional cues as well as target recognition signals should be provided to the regenerating axons. At present, however, almost no information is available in the literature on these mechanisms in adult, injured model systems.

Nogo-A Neutralization Improves Regeneration and Promotes Plasticity in Animal Models of Spinal Cord Injury and Stroke

After blockade of the Nogo-A pathways, two types of anatomical repair phenomena were observed: enhanced axonal sprouting and increased regeneration (Fig. 20.2b). *Regenerative sprouting* can frequently be observed after fiber tract lesions, as collateral sprouting close to the lesion site or from the injury site directly. If these fibers elongate over longer distances (beyond 1 mm) they are considered as regenerating axons (*regenerative growth*). In the injured spinal cord such axons can grow around the lesion and scar area and extend beyond the lesion site toward potential targets in the lower spinal cord. Fiber numbers often drop off at >2–5 mm, but regenerating axons covering >10 mm could also be observed. Corticospinal tract (CST) and

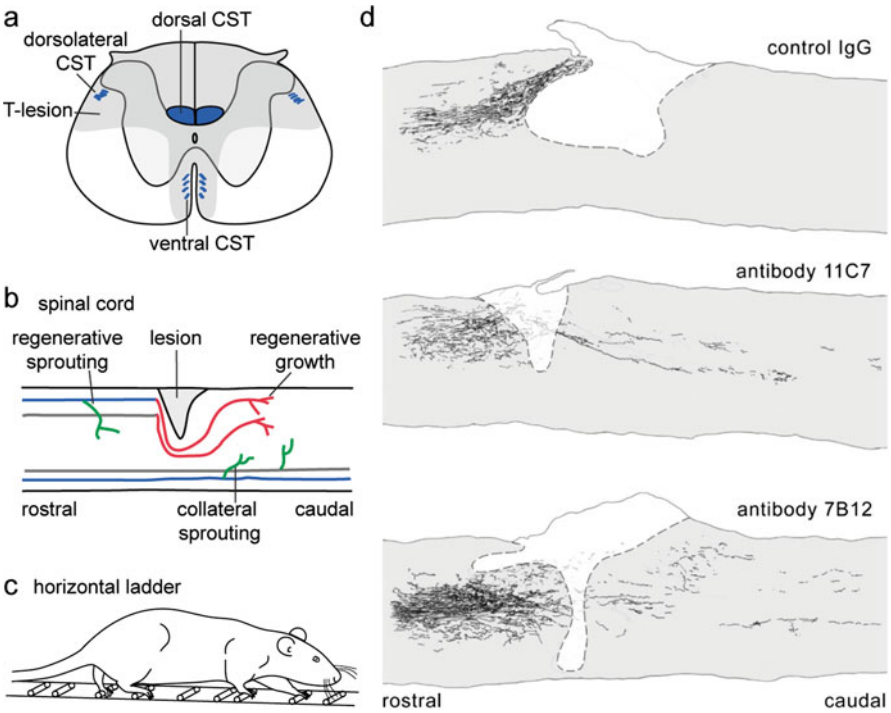

Fig. 20.2 Anatomical and behavioral aspects of Nogo-A signaling inhibition in spinal cord injury. (**a**) Scheme of a rat spinal cord cross-section depicting localization of the dorsal, dorsolateral, and ventral corticospinal tracts and a T-shaped transection (*gray shaded area*) which interrupts all of those tracts and is a widely used model in experimental spinal cord injury research. (**b**) Scheme illustrating axonal regeneration versus sprouting after spinal cord injury: regenerating axons originate from the cut end of injured axons (*red*); however, the shafts of damaged axons and preexisting, noninjured, spared fibers sprout also in response to the injury (*green*). (**c**) After spinal cord injury, the irregular horizontal ladder test is widely used for the assessment of locomotion and fine motor control in rats. (**d**) Camera lucida reconstruction of the spinal hemicord with regenerating corticospinal fibers rostral and caudal to the lesion site (light area) in control IgG and anti-Nogo-A antibody (mAb 11C7 and mAb 7B12)-treated rats. In the anti-Nogo-A antibody-treated rats, corticospinal fibers regenerated over ventrolateral tissue bridges into the caudal spinal cord. This regenerative growth was absent in the control antibody-treated animals. Reprinted with permission from Ann Neurol [27]

serotonergic fibers were studied most extensively. Additionally to injured axons, the noninjured, spared fibers can respond to the injury with sprouting, a phenomenon often called *compensatory sprouting* (Fig. 20.2b). In experimental stroke, recovery is predominantly mediated by compensatory collateral sprouting of the uninjured tracts.

Depending on the type of CNS injury, complete or incomplete, long distance regeneration or local sprouting could be more relevant for the functional recovery.

Suppression of the Nogo-A Pathway in Animal Models of Spinal Cord Injury

Nogo-A Neutralization by Antibodies

As reviewed in Table 20.1, multiple in vivo studies were carried out in SCI animal models to explore the future possibility of clinical studies with Nogo-A/NgR1 receptor blocking agents.

In the early 1990s, Schnell and Schwab [7] reported that the delivery of the monoclonal antibody called IN-1, an IgM recognizing and neutralizing Nogo-A, through implantation of antibody producing hybridoma cells in the brain increased the regeneration of cut axons past the lesion site in an incomplete rat SCI model. This experiment with IN-1 treatment has been repeated in multiple species (rat and marmoset monkey) and lesion paradigms, and demonstrated increased functional recovery as assessed by various locomotor tests (Basso, Beattie, and Bresnahan (BBB) test, narrow beam, horizontal ladder (Fig. 20.2c), etc.) [65–71]. Highly purified, new monoclonal antibodies directed against the Nogo-A-specific domains of the rat protein (11C7 and 7B12 antibodies) allowed repeating these experiments in clinically more relevant settings. These antibodies were delivered intrathecally via an osmotic minipump for 2 weeks, starting immediately after the injury. After mid-thoracic T-lesion (Fig. 20.2a), anti-Nogo-A antibody-treated animals had a much higher number of regenerating CST and serotonergic fibers (Fig. 20.2d) and consistently showed substantial functional recovery associated to this regenerative improvement in rats and macaque monkeys [27, 72–75].

As most of these in vivo studies were performed with acute antibody delivery (starting at the time of the SCI), delayed anti-Nogo-A antibody treatment regimens were also tested. These experiments showed that delaying the antibody delivery in rats by 1 week was still efficient in increasing CST axon regeneration and hindlimb recovery, whereas a delay of 2 weeks until the treatment start led to poorer outcomes [76, 77]. Combination of Nogo-A neutralization with several pharmacological treatments led to even more enhanced anatomical reorganization and functional improvements. The effects of anti-Nogo antibody treatment were strengthened by simultaneous neurotrophin-3 (NT-3) and NMDA-receptor 2d (NR2d) subunit delivery, which promotes neuronal growth capacity, survival, and synaptic plasticity, respectively [78, 79]. CSPGs and peri-neural net proteins exert additional inhibition on axonal growth. Combined treatments reducing CSPGs by chondroitinase ABC (ChABC) and blocking Nogo-A protein by antibodies were found to be beneficial for CST sprouting, regeneration, and for the recovery of forelimb functions after high cervical contusion lesions [80].

Anti-Nogo-A antibodies bind to cell membrane Nogo-A and may lead to internalization of the protein–antibody complex eventually leading to downregulation of Nogo-A tissue levels [81]. Additionally, due to the large size of the antibodies, steric blockade of the Nogo-A-Nogo receptor interaction may be a mechanism of action for Nogo-A neutralization.

Table 20.1 Key in vivo studies on Nogo-A signaling inhibition in spinal cord injury

Treatment	Study	Species age gender	SCI model	Route of administration	Anatomical readout and effect	Functional readout and effect
Anti-Nogo-A mAb IN-1	Bregman 1995 [65]	Rats, 6–8-weeks-old, Lewis (150–200 g)	Over-hemisection (right), T 6	Hybridoma cells secreting IN-1 Ab into the CSF at the time of the SCI	Up to 9 weeks post-SCI, increased CST (up to 7–11 mm, BDA tr.), serotoninergic and noradrenergic fiber growth (immunostaining)	After 4–6 weeks recovery, 80 % of IN-1-treated rats recovered contact placing responses, and stride length (foot-print analysis)
NEP1-40 (Nogo-66 antagonistic peptide)	Li and Strittmatter 2003 [83]	Mouse, 8–10-week-old, C57BL/6	Dorsal over-hemisection, T 6–7	Subcutaneous NEP1-40 treatment at the time, 4 h or 7 days after SCI	3–4 weeks past SCI dCST sprouting (BDA tr.) significantly increased both in acute and delayed NEP1-40 treatment groups. BDA/synaptophysin double-labelings suggest synapse formation	BBB score increase at 17 or 28 days post-SCI (acute or delayed, respectively). Additional foot-print and grid-walk improvement
NgR1(310)ecto-Fc (soluble function-blocking NgR1 ectodomain)	Li 2004 [29]	Rats, female Sprague–Dawley (190–250 g)	Dorsal over-hemisection, T 6–7	Intrathecal NgR1(310) ecto-Fc delivery by osmotic minipump for 4 weeks	Increased axonal sprouting of dCST (BDA tr.) and raphespinal fibers (immunostaining). Increased occurrence of BDA or 5-HT-synaptophysin double-labeled varicosities	Improved spinal cord electrical conduction and BBB scores at 14 and 28 days past SCI. Additional foot-print and grid-walk improvement
Anti-Nogo-A mAb 11C7 and mAb 7B12	Liebscher 2005 [27]	Rats, 3-months-old, Lewis	Half dorsal transection (T-lesion), T 8	2 weeks of intrathecal catheter delivery of purified anti-Nogo-A monoclonal IgGs	9 weeks after SCI, CST sprouting and regenerating fiber numbers at 0.5, 2 and 5 mm caudal to the lesion were significantly higher in anti-Nogo-A antibody-treated animals (BDA tr.)	Improvement in BBB score and various motor tasks, sensory cortex activation (contralateral hindpaw stimulation), absence of dysfunctions (14–55 days post-SCI)

Anti-Nogo-A mAb 7B12 and mAb h-Nogo-A	Freund 2006 [72]	Macaque monkeys of either sex	Lateral hemisection, C 7–8	Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 4 weeks at the time/site of SCI	Increased cumulative arbor length of BDA traced CST fibers caudal to the lesion in anti-Nogo-A Ab-treated animals	Improved manual dexterity ("modified Brinkman board," "reach and grasp drawer")
Anti-Nogo-A mAb 11C7 with or without parallel treadmill training	Maier 2009 [92]	Rats, female Sprague–Dawley (200–250 g)	Half dorsal transection (T-lesion), T 8	Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 2 weeks at the time/site of SCI	Both trained and nontrained 11C7-treated groups displayed increased CST fiber number caudal to the lesion (BDA tr.) and higher 5-HT fiber density in lamina VII	Tested by bipedal stepping, 9 weeks post-SCI, nontrained 11C7-treated rats demonstrated consistent step cycles, low paw drags and improved coordination. Parallel Ab treatment and treadmill training worsened the stepping qualities
Anti-Nogo-A mAb 11C7	Gonzenbach 2012 [77]	Rats, 8–10-week-old, female Lewis (180–200 g)	Half dorsal transection (T-lesion), T 8	Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 2 weeks at the time, 1 week or 2 weeks delayed past SCI	10 week post-SCI higher numbers of labeled CST fibers (BDA tr.) in the acute and 1 week delayed treatment groups, but not in the 2 weeks delayed Ab application	Increased BBB scores, swimming and narrow beam recovery in acute and 1 week treatment group, but not in the 2 weeks-delayed group. Reduction of muscle spasms

(continued)

Table 20.1 (continued)

Treatment	Study	Species age gender	SCI model	Route of administration	Anatomical readout and effect	Functional readout and effect
Anti-Nogo-A mAb (11C7) + chondroitinase ABC (ChABC)	Zhao 2013 [80]	Rats, adult male Lister hooded (150–200 g)	Dorsal column lesion, C 4	Acutely intrathecally 11C7 (2 weeks), delayed ChABC injection and infusion from 3 weeks (10 days), training from 4 weeks	11C7: increased growth of axons with diameter of >3 μm, ChABC: finer axons with varicosities, combinational treatment: highest sprouting and regeneration (BDA tr.)	Increased forelimb function in skilled paw reaching task (Montoya's staircase) in the combinational therapy group
Humanized NgR1-Fc decoy protein (NgR1(310)-Fc)	Wang 2014 [86]	Rats, 10–11-weeks-old, female Sprague–Dawley (220–240 g)	Moderate contusion injury, T 7 (10 g, 25 mm)	Intracerebroventricular infusion (continuous) or bolus delivery to the lumbar intrathecal space (once in every 4 days)	8 weeks postinjury, increased the fiber density of raphespinal axons caudal to the SCI (5-HT immunostaining)	Lumbar bolus dosing schedule promoted locomotor recovery from SC contusion as effectively as continuous infusion in open field (increased BBB scores) and grid-walking tasks

Abbreviations: 5-HT serotonin, *BBB* Basso, Beattie, and Bresnahan locomotor activity test, *BDA tr.* biotinylated dextran amine tracing, *C* cervical, *CST* corticospinal tract, *ChABC* chondroitinase ABC, *dCST* dorsal CST, *mAb* monoclonal antibody, *SCI* spinal cord injury, *T* thoracic, *wk* week

Blockade of Nogo-A Receptors and Signaling

Another approach to block Nogo-A function is to block the activation of the NgR1 receptor complex with a function-blocking peptide (NEP1-40) or soluble NgR1-Fc proteins. One advantage of this approach is the possible simultaneous blockade of several myelin-associated inhibitory NgR1 ligands such as Nogo-A, MAG, and OMgp, eventually even CSPGs [82]. Both acute and delayed intrathecal, subcutaneous, or intraperitoneal delivery of NEP1-40 was reported to increase the regeneration of both corticospinal and raphespinal fibers and lead to increased BBB locomotor activity scores and grid-walk performance [28, 83, 84]. Experiments applying the NgR1(310)ecto-Fc (soluble function-blocking NgR1 ectodomain) intrathecally or intracerebroventricularly led to a similar increase of regeneration and sprouting of corticospinal and serotonergic fibers together with increased locomotor recovery [29, 85, 86]. The studies by Wang et al. in 2011 and 2014 were performed with spinal cord contused rats, applying NgR1(310)ecto-Fc treatment in a close-to-reality SCI model. Triple therapy combining NgR1(310)ecto-Fc, ChABC for degrading CSPGs and peripheral nerve preconditioning injury increased the intrinsic growth potential of dorsal root ganglion neurons and allowed axons to regenerate millimeters past the spinal cord injury site [87].

Although only shown by a single study, blocking LINGO-1, a co-receptor of NgR1, by soluble LINGO-1-Fc fragments also resulted in elevated rubrospinal and corticospinal fiber sprouting and led to improved functional recovery in the cylinder test and in BBB test [30].

Multiple studies showed that inhibition of Rho-A/ROCK signaling leads to increased axonal regeneration and improved functional recovery. The C3 transferase or Y-27632 [88], Cethrin (BA-210) [89, 90], or fasudil [91] treatments led to similar enhancement of anatomical reorganization and behavioral improvements.

Suppression of Nogo-A Signaling in Animal Models of Stroke

Nogo-A Neutralization by Antibodies

Several successful animal studies with Nogo-A signaling inhibition in experimental stroke models are currently raising hopes for a neurorestorative stroke therapy (see Table 20.2 for summary). Neutralization of Nogo-A in rats with different blocking antibodies (IN-1, 11C7, 7B12) after large, cortical ischemic strokes has been shown to promote functional and anatomical recovery [93–95]. Lesion size was unaffected by the anti-Nogo-A antibody treatment [94]; the effects were therefore not due to increased neuroprotection. The large strokes in rats were triggered by the permanent occlusion of the middle cerebral artery or by photothrombosis, destroying most of the sensorimotor cortex and leading to major and permanent deficits in a fine motor control as shown, e.g., by pellet grasping or the error rate during ladder crossing (Fig. 20.3b, c). The anti-Nogo-A antibody treatment started either acutely after

Table 20.2 Key in vivo studies on Nogo-A signaling inhibition in stroke animal models

Treatment	Study	Species age gender	Stroke model	Route of administration	Anatomical readout and effect	Functional readout and effect
Anti-Nogo-A mAb IN-1	Papadopoulos 2002 [93]	Rats, adult male Long-Evans, 6–8-weeks-old, 200–300 g	Permanent occlusion of the middle cerebral artery (MCAo)	Hybridoma cells secreting IgM IN-1 Ab into the CSF at the time of MCAo	BDA tracing of contralesional forelimb motor cortex. Increased midline sprouting of corticorubral fibers	Single pellet grasping, recovery up to 80 % of baseline after 8 weeks, comparing to 50 % in control groups
Anti-Nogo-A mAb 7B12	Wiessner 2003 [100]	Rats, adult male Fischer F344, 300 g	Photothrombotic stroke of the sensorimotor cortex	Intracerebroventricularly (i.c.v.) by minipump, contralesional hemisphere, 24 h after stroke for 2 weeks	BDA tracing. Increased midline sprouting of intact corticospinal fibers at the cervical level	Montoya's staircase test, recovery to 70 % of baseline after 6–9 weeks from photothrombotic stroke or 7–12 weeks from MCAo
		Spontaneously hypertensive rats (SHR), adult males, 220–300 g	Permanent MCAo			
NgR1−/− or Nogo-A/B−/−	Lee 2004 [106]	Mice, adult male C57BL6, 8–10-week-old	Photothrombotic stroke of the sensorimotor cortex	Genetic knockout	BDA tracing. Increased midline sprouting of intact corticorubral and corticospinal fibers at the cervical level	Montoya's staircase test, NgR1−/− recovered up to 65 % and Nogo-A/B−/− up to 80 % of baseline after 30 days. Rats recovered to 70 % of baseline after 4 weeks. Improvement in the rotarod test
Decoy receptor NgR1(310) Ecto-Fc		Rats, adult male Sprague–Dawley, 250–350 g	Permanent MCAo	i.c.v. by minipump, contralesional, 1 week after stroke for 8 weeks		
Anti-Nogo-A mAb 7B12	Markus 2005 [94]	Aged rats, 25-months-old	Permanent MCAo	i.c.v. by minipump, contralesional, 1 week after stroke for 2 weeks	No difference in the lesion volume as assessed by MRI at acute and chronic time points	Single pellet grasping, recovered to 70 % after 14 weeks, when controls reached 40 %. By fMRI, increased thalamic activation in the anti-Nogo-A group
Delayed Anti-Nogo-A mAb 11C7	Tsai 2011 [99]	Rats, adult male Long-Evans (300 g)	Permanent MCAo	i.c.v. by minipump, contralesional, 9 weeks after stroke for 2 weeks	BDA tracing of contralesional forelimb motor cortex. Increased midline sprouting of corticorubral fibers	Single pellet grasping, recovery to 78 % of baseline after 18 weeks

Anti-Nogo-A mAb 11C7	Lindau 2014 [95]	Rats, adult female Long-Evans (230–400 g, 3–7-months-old)	Photothrombotic stroke of the sensorimotor cortex, lesion of >90 % of the forelimb motor cortex	Intrathecally through catheter at L 2 by minipump, at the time of stroke for 2 weeks	Retrograde and anterograde tracings. At 3 weeks in 11C7 treated group increase in bilaterally projecting cells. After 12 weeks strong increase in ipsilateral projecting cells. Increased number of midline-crossing fibers and ventral sprouts at the cervical spinal cord	Single pellet grasping, the 11C7-treated group recovered to 65 %±9 % of baseline. Control Ab group reached 24 %±6 %. In 11C7 group strong increase in responses of affected forelimb to ICMS of contralesional cortex. Pyramidotomy after recovery reinstated the deficit
Anti-Nogo-A mAb 11C7 with parallel or sequential single pellet grasping training	Wahl 2014 [96]	Rats, adult female Long-Evans (200–250 g, 3–4 old)	Photothrombotic stroke of the sensorimotor cortex, lesion of >90 % of the forelimb motor cortex	Intrathecally through catheter at L2 by minipump, Ab at the time of stroke for 2 weeks with the parallel training (100 reaches per day) starting 2 days after stroke or sequential training starting 2 weeks after the stroke	BDA tracing of contralesional forelimb motor cortex. The highest number of the midline-crossing CST fibers in the cervical spinal cord was detected in the sequential group. The parallel group showed extensive branching and aberrant innervation of the dorsal horn by the midline-crossing fibers	Single pellet grasping, anti-Nogo-A sequential group recovered to 86.3 %±2 % of baseline; parallel group to 10 %±5.2 %. Sequential group was best in the staircase and horizontal ladder tests. Inactivation of the midline-crossing fibers by pharmacogenetics in the sequential group reinstated the deficit

Abbreviations: Ab antibody, *BDA* biotinylated dextran amine, *CST* corticospinal tract, *ICMS* intracortical microstimulation, *i.c.v.* intracerebroventricularly, *mAb* monoclonal antibody, *MCAo* middle cerebral artery occlusion, *L 2* lumbar level 2, *wk* week

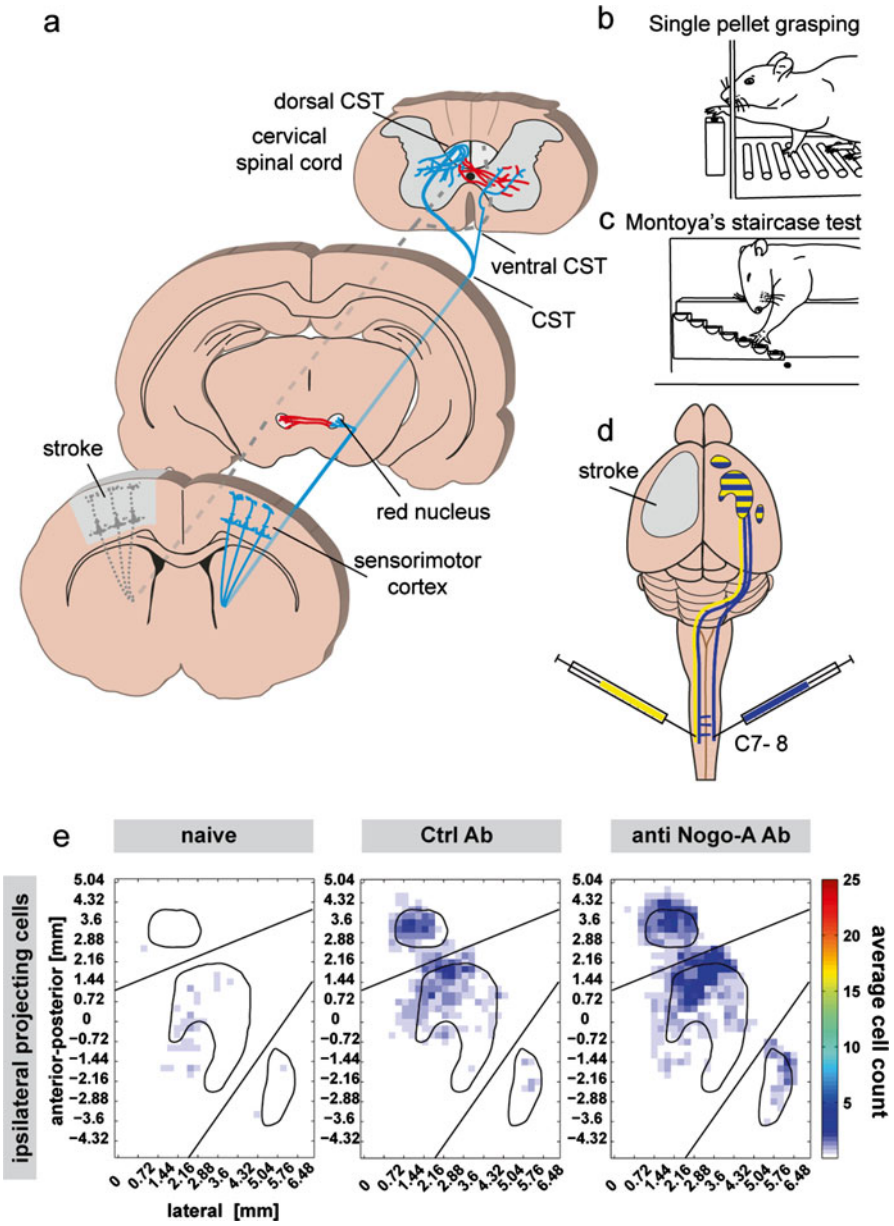

Fig. 20.3 Anatomical and behavioral aspects of Nogo-A signaling inhibition in an animal model of stroke. (**a**) Scheme of the compensatory sprouting of intact corticorubral and corticospinal fibers across the spinal cord and brainstem midline after large strokes destroying the sensorimotor cortex in rats which received anti-Nogo-A antibody; sprouting fibers are depicted in red. (**b**) After large cortical strokes, defects, and functional recovery of the fine motor control of the forelimb can be detected in the single pellet grasping task or (**c**) in the Montoya's staircase task in which rats

stroke [93, 95, 96] or 1 week [94, 97, 98] or 9 weeks after the stroke [99] led to an improvement of skilled forelimb functions to up to 70–80 % of the original performance before the stroke. Control antibody-treated groups did not recover above 30–50 %. As shown in several studies using anterograde axonal tracing techniques, this recovery was mediated by the intact, contralesional hemisphere from which corticorubral [93, 97, 99] and corticospinal [95, 96, 100] axons sprouted across the midline to the denervated red nucleus or cervical spinal cord, respectively (Fig. 20.3a). This rewiring of the intact CST was elegantly addressed by Lindau and colleagues [95] who demonstrated that in the first weeks after the stroke, the intact CST sprouts to the denervated cervical hemicord, but still preserves its original projection, leading to the occurrence of mirror movements in this time period. However, these preexisting connections are eliminated 3 weeks after the lesion. Preexisting ipsilateral corticospinal projections, located in rats mainly in the ventral column, were also shown to sprout under the influence of anti-Nogo-A antibodies, contributing together with the midline crossing fibers to the increased innervation of the stroke affected cervical spinal cord (Fig. 20.3a, d, e).

A recent study in a rat photothrombotic stroke model showed that sequential treatment, first with anti-Nogo-A antibody for 2 weeks followed by intensive forelimb grasping training was strongly beneficial, whereas simultaneous anti-Nogo-A treatment with intensive training starting directly after the injury is detrimental for the functional recovery [96]. Based on these results, we hypothesized that after stroke and probably also after SCI [92], the adult, injured CNS circuits need time for growth and rearrangement before the functionally meaningful connections can be stabilized by activity dependent mechanisms. Therefore, designing the optimal treatment paradigm and timing will be of utmost importance.

While most of the stroke studies focused on sensorimotor recovery, Nogo-A neutralization in the rat stroke or cortical lesion models was also shown to lead to cognitive improvements [101, 102].

Apart from extensive studies in rodent stroke models, there are also studies on macaque monkeys with a small excitotoxic lesion to the hand representation in the primary motor cortex; they show tendencies for improved recovery of manual dexterity in the anti-Nogo-A-treated group [103–105].

Fig. 20.3 (continued) have to grasp and eat sugar pellets. (**d**) Scheme of the two-color retrograde tracing from the cervical spinal cord used in (**e**) after a large cortical stroke. Contralaterally projecting cells are labeled in yellow and ipsilaterally projecting cells in blue. (**e**) Ipsilaterally projecting corticospinal neurons are very rare in intact rats. Sprouting and midline crossing of corticospinal fibers from the intact cortex to the denervated side of the spinal cord are enhanced by anti-Nogo-A antibody treatment 12 weeks after a large cortical stroke. The figure depicts false color-coded heat map (dorsal 2D view) of cell densities of recrossing or sprouting pre-existing, ipsilaterally projecting cells in the contralesional cortex in relation to the bregma (0 mm anterior–posterior and mediolateral). The straight lines indicate the boundaries of the rostral forelimb (rFL), the caudal forelimb area (cFL), and the secondary somatosensory cortex (S2). Modified from [95] and reprinted with permission from Brain

Suppression of Nogo-A Receptor Activation and Signaling

Inhibition of Nogo-66 binding to NgR1 by the soluble decoy receptor NgR1(310) ecto-Fc or by genetic knockout of NgR1 or Nogo-A/B after stroke led to increased recovery of skilled forelimb functions and motor coordination as well as to sprouting of contralesional, intact-side corticorubral, and corticospinal fibers [106]. Functional improvements were also obtained in stroke rat models after treatment with the NgR1 blocking peptide NEP1-40 [107]. Much less is known about the possible therapeutic importance in stroke of the recently identified Nogo-A receptor S1PR2. One study reported that intraventricular application of the S1PR2 antagonist, JTE-013, 2 days after stroke increased migration of endogenous neuronal progenitor cells toward the infarct site [108]. Several studies addressed the therapeutic efficacy of ROCK1/2 inhibitors in rodent models of stroke (for review, see [109]), but their main mode of action may be associated with vasodilation during and acutely after stroke causing a decreased infarct volume [110, 111].

Nogo-A in Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis is a neurodegenerative disease leading to progressive denervation of skeletal muscles, degeneration of spinal and cranial motoneurons, and consequential muscle paralysis. High expression of Nogo-A was observed in skeletal muscles of ALS patients and in mouse models at an early phase of the disease [112, 113]. Whether the increased expression of Nogo-A by muscle fibers may be a common feature with other neuromuscular diseases and reflect the muscle denervation, remains unclear [114, 115]. However, systemic administration (i.p.) of an anti-Nogo-A antibody or genetic deletion of Nogo-A in a mouse model of ALS delayed disease progression [116, 117]. The ectopic expression of Nogo-A in intact, wild type muscle cells caused the retraction of motor axons and the disassembly of neuromuscular junctions suggesting that the ectopic high levels of Nogo-A can induce motor nerve terminal retraction [117]. Inside the motoneurons, on the other hand, the intracellular Nogo-A and B expressed in the endoplasmic reticulum can play a neuroprotective role [118], suggesting that the muscle-derived and neuronal Nogo-A may have opposite roles in ALS.

Nogo-A in Multiple Sclerosis (MS)

Multiple sclerosis is an inflammatory disease featuring demyelination, oligodendrocyte loss, and axonal degeneration. Nogo-A was postulated to play roles in remyelination, lack of regeneration, and perhaps also in inflammatory events during the course of MS [119]. The occurrence of anti-Nogo-A auto-antibodies was reported in serum and cerebrospinal fluid (CSF) of MS patients and acute neurological

disease patients, but not in healthy individuals [120]. In the mouse experimental autoimmune encephalomyelitis (EAE) model, immunization with a Nogo-A-specific peptide (aa 623–640) [121] or with peptides from the Nogo-66 region [122] was protective by shifting the T-cell profile toward beneficial Th2 cells. However, the immunization with Nogo-66 peptides was mildly encephalitogenic in susceptible mouse strains. Nogo-A could act directly on immune cells expressing NgR1 [123]. However, the deletion of NgR1 and 2 did not affect the profile of Th-cells in the EAE model [124]. Deletion of Nogo-A/B/C [121, 122], NgR1 [125], or LINGO-1 [126] and silencing of Nogo-A by systemic siRNA application [127] as well as systemic treatment with anti-Nogo-A (i.v.) [121, 125] or anti-LINGO-1 (i.p.) [126] antibodies strongly decreased the severity of EAE. Interestingly, a recent study links an elevated expression of S1PR2, which can function as a Nogo-A signal transducing receptor, in females to their increased susceptibility to MS and EAE [128]. Systemic pharmacological interventions targeting Nogo-A signaling in EAE models and in MS patients have a potential to act not only in the periphery by modulating immune responses, but also to penetrate through the leaky blood–brain barrier into the CNS and to promote neuronal regeneration or remyelination [34].

Preparing Translation: Preclinical Studies for Nogo-A Blocking Agents

The significant number of studies with Nogo-A/NgR1 signaling suppression paved the path to clinical studies that are currently being carried out or are planned in CNS injured, MS, and ALS patients [129].

Preclinical spinal cord and cortical lesion studies with anti-Nogo-A antibodies were conducted both on rodents and on primates. As the motor system organization, the tissue reaction in response to injury and the behavior of nonhuman primates resemble the human situation; these studies were an important step for the clinical translation [130]. The efficacy of the anti-Nogo-A antibody treatment was confirmed by the studies on macaque monkeys and showed substantial recovery of hand dexterity, as well as sprouting and regeneration of CST axons without signs of pain or other side effects [72, 74]. Toxicological studies with the clinical, human anti-Nogo-A antibody were also carried out on two species, rats and primates.

The severity, location, and extent of spinal cord injury or stroke define the possible treatment options and their efficacy. In complete spinal cord-injured patients, for example, in addition to growth-promoting interventions, bridges would be required to allow axotomized fibers to cross the lesion site [131, 132]. In cases of anatomically incomplete lesions, regenerating fibers are able to bypass the injury site when supported by tissue bridges and pharmacological treatments, e.g., anti-Nogo-A, CSPG degradation, or stimulation of intrinsic neuronal growth mechanisms. The damage after spinal cord injury in humans often occurs in the ventral spinal cord frequently affecting more than one spinal segment, whereas in the experimental models the spinal cord is usually lesioned by a dorsal approach with a well-defined transection

or small contusion. Studies with clinically more relevant lesions such as large compressions and contusions would be valuable for direct comparison with clinical cases [133]. A recent study in spinal cord contused rats treated with humanized NgR1(310)-Fc showed that regeneration and locomotor recovery were promoted [86]. Optimal treatments may need to be defined depending on whether long-distance regeneration or local sprouting of injured or intact fibers is more desirable in a lesion with given location and extent.

For many of the animal experiments with Nogo pathway blockers, acute treatment regimens were applied. Delayed delivery of both Nogo-A and NgR1 neutralizing agents were tested in rat models of spinal cord injury; the efficiency of the treatments was good when started at 1 week after injury, but declined at 2 weeks after injury [77, 86]. Interestingly, in stroke models, delayed treatment was effective even when started 9 weeks after the infarct [99]. For combined pharmacological and rehabilitative training therapy the timing and the optimal time windows need to be taken into account. Parallel intensive treadmill training together with anti-Nogo-A antibody treatment in the first 2 weeks after a spinal cord lesion resulted in very poor functional recovery despite of a strong regenerative fiber response [92]. A similar result was seen after stroke. However, a sequential treatment, first with the antibody followed in time by rehabilitative training was strongly beneficial [96]. It is probable that the injured CNS goes through an initial phase of sprouting and plasticity which can be enhanced by Nogo-A neutralization. Subsequently, the newly formed connections have to be stabilized and functionally meaningless connections have to be pruned by activity-dependent processes, which can be enhanced by intense rehabilitative training.

An important point concerns the best route of application of a drug. The blood–brain barrier prevents or severely restricts access of many compounds, including antibodies, to the intact CNS [134]. At lesion or inflammatory sites, however, the blood–brain barrier is open temporarily, allowing antibodies to penetrate into the surrounding CNS tissue. In most of the spinal cord injury and stroke preclinical experiments, a direct intrathecal way of application of antibodies, peptides, or fusion proteins was chosen. By lumbar subdural catheter infusion with osmotic minipumps, high levels of drugs can be delivered over 2–4 weeks. After intrathecal infusion, antibodies are distributed by the CSF circulation and therefore can reach not only spinal cord, but also brain in rats and monkeys [81].

Translating in vivo experiments to clinical studies faces the challenge of comparable and relevant functional outcome measures. For rats and higher vertebrates, a detailed kinematic gait analysis, which is modeled after the human analysis techniques [135], should replace the current simple, widely used, but subjective and nonlinear locomotor scores [136]. Objective and quantitative assessments for hand function, balance, or bladder function are being developed for experimental animals. Clinically, neurophysiological assessments (motor- and somatosensory-evoked potentials, fMRI, TMS) also play important roles; many of these testing methods have been successfully used in animals [137]. Additionally, the standardization of diagnosis and functional assessment protocols is of high importance. Clinical data are collected in databases (EMSCI, European Multicentre Study about Spinal Cord

Injury; NACTN, North American Clinical Trial Network) and serve as valuable data bases for the design and as historical controls for future clinical studies.

The deficits of the bladder, bowel, and sexual function, and complications like chronic pain or spasticity have a strong impact on the life quality of spinal cord-injured patients. These functions have not been extensively addressed in the pre-clinical studies. Anti-Nogo-A antibody-treated rats with partial spinal cord transections regained autonomous bladder function 7–9 days earlier than control antibody-administered rats. Furthermore, in these animals the pain threshold was not altered [27], and the occurrence and severity of spastic cramps were decreased [76, 77], suggesting that the treatment led to the stabilization of functionally correct circuits. Importantly, none of the studies with Nogo-A suppressing treatments reported pain, increased spasticity, discomfort, or behavioral disturbances associated to the therapy [26].

There is also a strong need for developing efficient neurorestorative treatments for patients with large strokes. The highest degree of spontaneous recovery in smaller strokes occurs in patients in the first 3 months after the stroke [138] and therefore timing the pharmacological treatment to this period, which was shown to be efficient in animal models [99], may greatly improve functional outcomes also for the larger lesions [139].

In the long run and for the repair of very large lesions, multiple approaches are going to be required in order to optimize regeneration and functional recovery. Alongside treatments which overcome growth inhibition by myelin and the glial scar, well balanced and timed stimulation of the neuronal growth program and implantation of artificial or cellular bridges could be used. These pharmacological treatments then should be coordinated with rehabilitative training to support the plastic changes in the CNS leading to functional recovery.

Interventions Blocking Nogo-A Signaling in Clinical Trials

Spinal Cord Injury

The first in-human anti-Nogo-A antibody Phase I clinical trial was conducted in several centers in Europe and Canada with 52 acutely spinal cord-injured patients suffering from severe para- and tetraplegia and was completed in September 2011 [140] (http://clinicaltrials.gov/ct2/show/NCT00406016). This study assessed the technical feasibility, safety, and pharmacokinetics of administering the fully human anti-Nogo-A human antibody ATI355 (Novartis Pharma) intrathecally to patients with acute spinal cord injury and confirmed AIS-A and B clinical classification. The antibody administration started at 4–28 days after the injury by either continuous intrathecal infusion for up to 28 days or in 6 intrathecal bolus injections over 4 weeks. Infusions and injections were made into the lumbar cerebrospinal fluid space. The ATI355 antibody was very well tolerated and no serious adverse events were reported. The bolus application had increased safety in comparison with

catheter-mediated infusions from external pumps. A multinational, multicenter, Phase II placebo-controlled clinical trial is currently in preparation to assess the efficacy of the treatment. This trial, which will be conducted in severe incomplete spinal cord-injured patients, requires optimized early diagnosis and sensitive, well-standardized outcome measures in all participating centers.

Ischemic Stroke

Small Phase I and II clinical trials with a humanized anti-myelin-associated glycoprotein (MAG) antibody (GSK249320, GSK) in healthy subjects and in stroke patients have been completed [141, 142]. The antibody was administered systemically (i.v.) and was reported to cross the blood–brain barrier [142]. No serious adverse events were recorded and modest trend toward improvement in the gait velocity was reported [142] (http://clinicaltrials.gov/ct2/show/NCT00833989).

A clinical study with the anti-Nogo-A antibody ATI355 (Novartis Pharma) in stroke patients is currently in preparation, as well as a trial with the soluble decoy NgR1 receptor (humanized NgR1(310)ecto-Fc; Axerion).

Amyotrophic Lateral Sclerosis (ALS)

A Phase I trial with humanized anti-Nogo-A antibody (GSK1223249, Ozanezumab, GSK) on 76 patients with familiar or sporadic forms of ALS has been completed [143]. This study assessed safety, pharmacokinetics, and functional effects of single or double intravenous dose of the antibody. Ozanezumab was well tolerated without severe adverse events related to the treatment. The efficacy of Ozanezumab treatment will be tested in a currently ongoing large Phase II multicenter trial in which patients with familial or sporadic ALS will receive i.v. infusion of the antibody or placebo every 2 weeks over 48 weeks. The trial will assess functional clinical outcomes and survival of ALS patients over the treatment period (http://clinicaltrials.gov/ct2/show/NCT01753076).

Multiple Sclerosis (MS)

Two Phase I clinical trials of i.v. treatment with Ozanezumab (GSK) in patients with the relapsing-remitting form of MS were conducted in Europe and Australia in 2011–2012 (ClinicalTrials.gov identifiers: NCT01435993 and NCT01424423). The first result shows that the antibody was well tolerated and no severe adverse events were reported.

Conclusions

In this chapter, we have discussed how Nogo-A function-blocking interventions could be relevant for enhancing repair processes and improving neurological status in spinal cord injury, stroke, amyotrophic lateral sclerosis, and multiple sclerosis patients. There are no cures available for most of these neurological conditions at present. In the long run, future therapies should incorporate multidisciplinary approaches by combining intrinsic neuronal growth-promoting treatments with interventions that render the inhibitory CNS environment (myelin and glial scar) more permissive for axonal regeneration and with rehabilitative training.

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Chapter 21
Intrinsic Neuronal Mechanisms in Axon Regeneration After Spinal Cord Injury

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Abstract Axon transection and failure of axon regeneration after spinal cord injury result in permanent functional deficits. Previous focus on blocking the inhibitory environment turns out to be insufficient to achieve robust axon regeneration in the central nervous system (CNS). Loss of intrinsic axon growth ability in adult CNS neurons might also play a critical role in underlying such regeneration failure. Based on recently revealed mechanistic insights about intrinsic ability controls, several experimental strategies have been devised to promote injured axons to regenerate with large quantities and long distance, and may provide important therapeutic approaches to recovering function after spinal cord injury.

Keywords Axon regeneration • Intrinsic • cAMP • PTEN • mTOR • SOCS3

Introduction

Normal function of the neuronal circuits in the spinal cord requires delicate control of the supraspinal structures in the brain. Spinal cord injury often results in interruption of such controls from the supraspinal neurons due to severing of their spinal-projecting axons (i.e., axotomy), leaving the cord caudal to the injury without proper function. In most cases following spinal cord injury, the supraspinal neurons largely survive with their axon stumps despite degeneration of their distal segments [1–3]. Thus, developing strategies to promote injured axons to regenerate and rebuild functional connections across the lesion site might be ideal for behavioral restoration in spinal cord patients.

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In principle, characteristic of a therapeutic method should be robust effect, good safety margin, and perhaps well-documented mechanisms. In light of these "criteria", here we will first discuss why it may be a good approach to explore intrinsic regenerative capacity of the adult CNS neurons which has long been underappreciated until recently. Then we will review the evidence supporting the loss of intrinsic regenerative ability of adult CNS neurons, and more importantly, examine our current understandings of the mechanisms. Finally, based on the examined evidence, we will propose potential strategies (and molecular targets, in some cases) that can enhance the intrinsic regenerative ability of adult CNS axons. We will also provide our perspective on such strategies regarding their potentials as well as complications.

Why Exploring the Intrinsic Mechanisms?

Defying a dogma that axons do not spontaneously regenerate in the adult mammalian CNS ever since Ramon and Cajal [4], seminar studies from Aguayo and his colleagues demonstrated that peripheral nerve grafts transplanted into the lesion site in adult CNS can allow some CNS axons to regrow into the peripheral nerve grafts [5–7]. As the peripheral nerve is regarded as a permissive substrate for axon growth, these observations have prompted a major hypothesis stating that the adult CNS neurons possess intrinsic ability to regrow their axons and that the reason for regeneration failure must be due to the inhibitory local environment in the CNS. Even since, the field has expanded quickly and has identified many extracellular inhibitors either present or induced by injury in the adult CNS, including a number of molecules associated with the central myelin, the astroglial scar, and the perineuronal net [8–15].

It appears that removing these inhibitory activities might promote compensatory axon sprouting and enhance the regeneration of some types of axons. For example, knocking out myelin-associated inhibitors could enhance compensatory collateral sprouting, but failed to trigger robust axon regeneration in vivo after spinal cord injury [16, 17]. Neutralizing the activity of PTP-sigma (a transmembrane protein tyrosine phosphatase), a receptor of chondroitin sulfate proteoglycans (CSPGs), promotes the regrowth of injured serotonergic axons, but not corticospinal tract (CST) axons [18]. Moreover, it is also important to recognize that in Aguayo's transplantation studies, only a limited subset of CNS axons were able to regrow into the permissive peripheral nerve grafts, while others failed to do so [6, 7, 19]. Thus, strategies of manipulating the growth environment alone may not be sufficient in achieving robust axon regeneration and thus possible functional recovery.

While certain environmental influences (e.g., cystic cavity and scar formed after spinal cord injury) on axon regeneration are clearly of great importance, it is difficult to image the neurons themselves as being a bystander. Thus, loss of intrinsic growth capacity of adult CNS neurons has been proposed to be a major reason for the regeneration failure [20–22]. During normal development, young CNS neurons at

early developmental stages grow their axons extensively to innervate their targets. Is it possible that the adult CNS neurons simultaneously lose their axon growth ability as they mature? What will happen to the immature neurons if they are experimentally presented with nonpermissive adult growth substrate? Will they fail to grow their axons if role of the environmental inhibition is so important, as many have previously thought? Moreover, it is conceivable that before the adult neurons decide how to respond to the axotomy, they need to first "sense" the initial insult. Could the axotomy itself further dampen neurons' intrinsic growth ability as one part of the otherwise intact neuron is suddenly "amputated"?

Loss of Intrinsic Axon Growth Ability in Mammalian CNS Neurons

Development-Dependent Mechanisms

One ideal paradigm to test developmental change of neurons' intrinsic ability to grow their axons is to compare the growth of CNS neurons from different developmental stages in the exactly same environment so that influence from the environment can be ruled out during comparison. In theory, this can be done in vivo by transplanting them into the same site in a host animal. However, this is technically challenging partly due to poor survival of the transplanted postnatal cells [23].

Experiments using ex vivo systems with hetero-chronic explant co-cultures turn out to rather informative. By co-culturing hamster retina tissue adjacent to tissue derived from their physiological brain target tectum, it was found that the retina from pups on or after postnatal day 2 was unable to extend axons into the co-cultured tectal targets even when the target tissue is embryonic. In contrast, embryonic retinal axons could regrow into the adjacent tectum derived from animals of any age, even overcoming the non-permissive environment in the adult tectum [21]. Such experiment nicely demonstrated a dramatic reduction in growth ability of hamster retinal axons around birth. Using similar tissue explants, dramatic developmental reduction of axon growth vigor has also been reported in other parts of mammalian brain including brainstem [24], cerebellum [25], and entorhinal cortex [26].

Culturing purified retinal ganglion cells (RGCs, regarded as CNS neurons) in a dish in a more "simplified" model, Goldberg and colleagues also found a precipitous decline in the vigor of axon outgrowth around birth: embryonic RGC axons extended ~10 times faster than the postnatal ones did [22]. Since such dramatic decline of growth power occurs around birth, right after the rodent RGC axons finish innervating their targets, one hypothesis proposes that the CNS neurons are developmentally programmed to switch off their growth programs, possibly by involving both genetic and epigenetic mechanisms, to avoid over-shooting their targets. Then, programs associated with events such synapse formation/maturation, dendritic growth [27] and neuron–glia interaction can be switched on to allow the developmental process to further proceed.

The molecular mechanisms for the development-dependent loss of axon growth ability are unclear. To make the link of a molecule or signaling pathway with such loss, it is suggested that one not only demonstrates the temporal correlation between regulation of the molecule/pathway and loss of axon growth ability during development, but also needs to convincingly "rescue" such loss by manipulating such molecule/pathway, ideally in vivo in mature adults. For mammalian CNS neurons, Bcl-2 (B-cell lymphoma 2) was among the first to be implicated in this process, as downregulation of the gene parallels the reduction of axonal growth ability during development [28]. Overexpression of Bcl-2 appears to increase axonal growth at least for immature RGCs, suggesting successful "rescue" of developmental loss of axon growth ability [28, 29]. However, overexpression of Bcl-2 fails to induce axon regeneration when axotomy occurs in more mature animals [30]. Moreover, the well-known prosurvival effect of Bcl-2 overexpression may confound its seemingly "proregenerative" effect observed for immature RGCs [22, 31].

Other important molecules/pathways contributing to development-dependent loss of axon growth ability include regulation of the members of the Kruppel-like factors (KLFs) [32, 33], downregulation of cyclic adenosine monophosphate (cAMP) [34, 35], and downregulation of the mammalian target (mTOR) of rapamycin [36, 37].

KLFs

The KLFs are a 17-member family of zinc-finger transcription factors. They are involved in regulation of cell-cycle exist and terminal differentiation of cells. The KLFs were first implicated in axon growth and regeneration in studies in zebrafish [38]. In another independent study in rodents, KLF4, one member of the KLF family, was identified as a potent inhibitor of axon growth for cultured hippocampal neurons in a screening involving more than 100 neuronal genes [33]. Importantly, the authors also found that targeted deletion of the KLF4 gene promoted regeneration of RGC axons in vivo in adults after optic nerve crush. Other members of the KLFs may also be involved, as it was found that while KLF4/9 were upregulated in adults, KLF6/7 were downregulated in comparison with embryonic RGCs. Indeed, overexpression of KLF7 has also been shown to promote axon growth after spinal cord injury in adult mice [32]. Thus, some KLFs seem to be important regulators of axon growth ability for CNS neurons.

cAMP

In characterizing the signaling mechanisms that controls the survival of rat RGCs in culture, Barres and other colleagues found that the highly purified postnatal rat RGCs could not survive well or extend their axons supported with a cocktail of peptide trophic factors, unless their intracellular cAMP was elevated either by electrical activity or pharmacological methods [39]. It is believed that by default the

CNS neurons may need trophic support to inhibit their tendency towards succumbing to apoptosis. However, the neurons do not extend their axons by default, even their survival is guaranteed by blocking apoptosis, unless signaled by trophic factors to do so [22]. Is it possible that the level of intracellular cAMP regulates the "outside-in" trophic signaling? Indeed, it was found that increasing intracellular cAMP could rapidly recruit the receptor tyrosine kinase TrkB, one type of trophic factor receptors, to the plasma membrane [40]. It was believed that cAMP might as well increase the amount of surface receptors for other trophic factors, thereby increasing the neuron's overall responsiveness to extracellular trophic factors [40]. These studies, along with other extensive studies on neuronal trophic signaling, provide important insights into understanding neuron's intrinsic ability to survive and to grow [41–45].

Is intracellular level of cAMP developmentally regulated? In rat RGCs, measurement in these CNS neurons freshly separated from the animal showed that RGCs of postnatal day 5 pups contained at least three times lower level of cAMP compared with embryonic day 18 RGCs and that the cAMP levels remained low in all postnatal RGCs up to adulthood [34]. Such developmental decrease of intracellular cAMP level parallels the developmental loss of RGC axons' growth ability in rats, as discussed previously.

Can application of exogenous cAMP promote axon regeneration for adult CNS neurons? Although the intracellular level of cAMP can be easily elevated using a membrane-permeable form of cAMP, effect of cAMP alone on axon regeneration appears to be limited for CNS neurons [46, 47]. This is despite the robust proregenerative effect induced by elevation of cAMP or activation of its downstream target cAMP response element binding protein (CREB) in sensory neurons [35, 48]. Nevertheless, when combined with application of exogenous trophic factors, elevation of cAMP can enhance axon regeneration for both CNS and sensory neurons [46, 49].

mTOR

mTOR (originally "mammalian" TOR, now officially "mechanistic" TOR), a serine/threonine protein kinase, is a master regulator involved in cell's responses to diverse environmental clues such as availability of energy and nutrients as well stress stimuli [50]. Many of cell's basic behaviors such as cellular growth and size are controlled by mechanisms involving mTOR, and activation of mTOR often leads to cell overgrowth in terms of the size and mass accumulation. It is thought by some that ability of axon growth may also be, at least partly, controlled by similar mechanisms involving mTOR. Indeed, in the mouse RGCs mTOR activity is very high during embryonic stage when cellular growth including axon extension is a major theme [37]. The endogenous level of mTOR becomes diminished in ~90 % of the adult RGCs, suggesting that mTOR activity is developmentally downregulated in correlation with completion of major cellular growth events. Deleting the gene encoding the tumor suppressor phosphatase and tension homolog (PTEN) increased mTOR activity for the axotomized adult RGCs and induced robust axon regeneration in those CNS neurons [37].

The protein mTOR can interact with several other proteins to form two distinct complex mTOR complex 1 (or mTORC1) and 2 (mTORC2). It was found that rapamycin, a selective inhibitor of mTORC1, blocked PTEN deletion-induced axon regeneration [37], suggesting the involvement of mTORC1. However, such experiment did not completely rule out the involvement of mTORC2 in axon regeneration and more studies are required to address the individual roles of mTORC1 and mTORC2 in axon regeneration.

Interestingly, it has been recently shown that the newly differentiated neurons from neuronal stem cells transplanted into the lesion cavity after a complete rat spinal cord injury are able to project their axons rather extensively into both sides of the host tissue—yet another manifestation of strong axon growth capacity of young neurons—and such axon growth can be partially blocked by mTOR inhibitor rapamycin [51, 52]. This raises a possibility that mTOR activity may play a role in axon growth in both young and adult neurons at of certain types.

Is direct activation of mTOR sufficient to induce axon regeneration for adult CNS neurons? It seems so, as genetic deletion of tuberous sclerosis complex 1(TSC1), a negative regular of mTOR, also increased mTOR activity in axotomized adult RGCs and induced axon regeneration [37]. Interestingly, axon regeneration appears less robust after deletion of TSC1 compared with that after deletion of PTEN, despite higher percentage of mTOR-positive RGCs. This observation seems to suggest activation of mTOR activity may not be sufficient on its own for robust axon regeneration for some adult CNS neurons. This also suggests that mTOR may be only one of many downstream effectors of PTEN deletion-induced axon regeneration. Identifying other effectors would not only elucidate the mechanism of induction of axon regeneration after PTEN deletion, but also provide more understandings of the signaling networks required for CNS axon regeneration in general.

Injury-Induced Mechanisms

Do all adult CNS neurons lose their intrinsic ability to extend their axons as they mature? Could some of them still retain some ability? Plasticity is well observed in intact mature CNS, which in theory requires certain forms of growth of axons, possible examples of which include growth of axon terminals associated with new synapse formation. So it is not completely impossible that a small subset of adult CNS neurons still retain certain intrinsic competence to grow their axons. For example, in intact mouse RGCs, ~10 % of them maintain their endogenous mTOR activities till adulthood. One may speculate that those mTOR-positive adult RGCs may possess certain axon growth ability.

If we assume that not all adult CNS neurons lose their intrinsic growth ability, then one may ask: why does failure of axon regeneration so catastrophically apply to all adult CNS neurons? In mouse adult RGCs, it would be difficult to miss the regeneration phenotype with current methodology if 10 % of them (i.e., ~4500 RGCs in number) could regrow their axons after axotomy (which is not true), as optic nerve injury has been so extensively studied. Is it possible that the “residual” intrinsic growth ability in intact adult neurons gets further lost soon after axotomy? In other

words, can axotomy itself further trigger reduction of adult CNS neurons' "residual" intrinsic growth ability? Or, any impacts of axotomy would be confined locally to the axonal stump leaving most part of the neuron including its cell body "untouched"?

Before attempting to address this question, we will first discuss what are the cellular events following axotomy. Soon after the proximal part of a typical CNS axon gets disconnected from its distal part, the axon stump dies back and then reseals the damaged membrane within hours or days. More local events then follow, eventually resulting in the formation of a dystrophic, dominant axonal bulb that stays for months or years, if the axotomized neuron does not die [53].

For the injured axon to regenerate in a sustainable fashion, the cell body would need to be "informed" of such injury via some retrograding signaling, so that a regrowth program can be activated to allow raw material to be synthesized in the cell body and transported to the growing tip of the axon. So, the injury-induced signals must be an integral part of the regeneration process [54]. Indeed, blocking retrograde signaling of injury by knocking down the dual leucine zipper kinase (DLK) significantly reduced the retrograde cell death but also compromised the extent of axon regeneration [55, 56]. So, the injury signals are not "all bad" for axon regeneration. But, they are not "all good" either.

Indeed, in the adult mouse RGCs, as early as 1 day after axotomy, the percentage of the mTOR positive RGCs was downregulated by 50 % (in addition to the development-dependent downregulation of mTOR described previously); by 1 week after injury, almost none of the RGCs are mTOR positive [37]. Such injury-induced mTOR downregulation is expected to further diminish the intrinsic growth ability of the RGCs. Similar injury-induced mTOR downregulation was also observed in the CST neurons after experimental spinal cord injury [36]. So, while a major part of the loss of intrinsic axon growth ability for adult CNS neurons may be development-dependent, certain to-be-defined injury signals derived from axotomy itself may further contribute to such loss, for example, by downregulating mTOR activity.

It is not clear whether axotomy can affect other developmental regulators of axon growth ability such as the KLFs and cAMP. Nor is it known how axotomy leads to decline of some positive regulators such as mTOR. Dissecting out different components ("bad" or "good") of the injury signal and then decoding its acting pathway will be another important aspect of understanding the failure and success of axon regeneration.

Mechanisms regulating the loss of intrinsic axon growth ability discussed above are summarized in Fig. 21.1a.

"Preconditioning" Effect in Sensory Neurons

Although we have been focusing on the intrinsic mechanisms of CNS neurons, it is noticeable that knowledge from studying the conditioning effect in the sensory neurons from dorsal root ganglia (DRG) has provided continuous insights and inspirations for many. Stemming from a unipolar axon, each DRG neuron contains a peripheral branch of axon that innervates the peripheral targets and a central branch

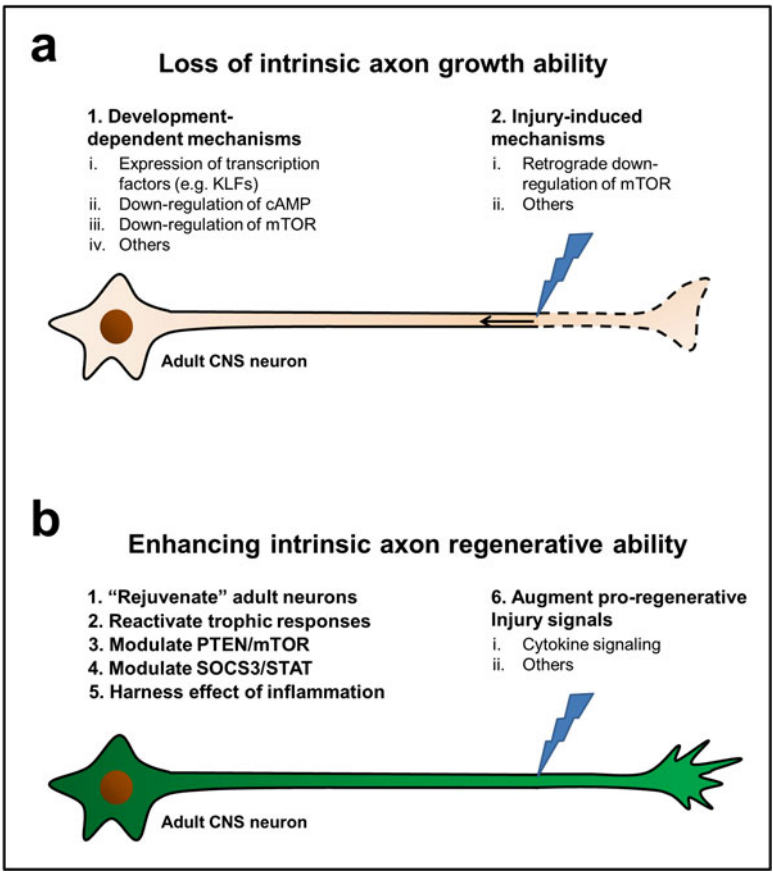

Fig. 21.1 Mechanisms that regulate loss of intrinsic axon growth ability and strategies of enhancing the intrinsic axon regenerative ability in adult CNS neurons

of axon that relays afferent inputs via the spinal cord to the supraspinal nuclei. After axotomy, whereas the peripheral branch can spontaneously regenerate, the central branch from the same DRG neuron cannot. Interestingly, if a "preconditioning" lesion such as sciatic nerve crush is applied at the peripheral branch, both the peripheral and central branches can now regenerate better in response to a second lesion [57, 58]. The fact that the preconditioned sensory neurons can regenerate their central branches of axons after spinal cord injury is particularly encouraging [57], as it provides a strong example that the inhibitory environment after spinal cord injury can be overcome by boosting the axonal regenerative ability of the neurons alone without disinhibiting the environment.

After the preconditioning lesion, injury-induced signals such as locally released cytokines interleukin-6 (IL-6) and ciliary neurotrophic factor (CNTF) can constitute important retrograde signaling that primes the DRG neurons [59–61]. Intracellular

pathways such as the janus kinase (JAK)–signal transducer and activators of transcription (STAT) pathway can then be activated in the cell bodies of DRG neurons that eventually switch on a regenerative program. Interestingly, the JAK–STAT pathway can also be targeted in the CNS neurons, as activation of such pathway by deletion of suppressor of cytokine signaling 3 (SOCS3) promotes axon regeneration in adult RGCs [62].

Another important mechanism underlying the preconditioning effect is the elevation of cAMP in the DRG neurons [35], the role of which in CNS axon regeneration has been discussed previously. Other salient mechanisms may well exist [63]. Exploring these mechanisms will continue to generate more understandings of axon regeneration after both peripheral and CNS axon injuries.

Strategies that Enhancing the Intrinsic Axon Regenerative Ability

For spinal cord injury patients, regenerating axons is vital in rebuilding the damaged neuronal circuits. In light of the criteria we've outlined at the beginning, as well as the recent progress the field has made (as discussed above), here we summarize the potential therapeutic strategies aimed at enhancing intrinsic axon regenerative ability of adult CNS neurons (also see Fig. 21.1b).

"Rejuvenating" Adult CNS Neurons with Transcription Factors

It may be possible to "reprogram" the adult CNS neurons in a way that push the neurons back to their developmental immature status to allow axon regeneration to occur. Given the progress made in the field of iPS cells (induced pluripotent stem cells) [64, 65], overexpression of certain transcription factors may work for certain adult CNS neurons. For the CST neurons, it is interesting that overexpression of KLF7 alone induced some regenerative sprouting [32]. It still waits to see whether more extensive axon regeneration can be achieved, for example, by overexpressing a combination of specific transcription factors.

Reactivating Trophic Responses with Growth Factors and cAMP

Exogenous growth factors have been applied to promote neuronal survival and axon regeneration with limited success [66]. As intracellular elevation of cAMP is shown to increase neuronal trophic responsiveness [40], combining application of growth factor and methods of elevating intracellular cAMP may be more effective in promoting axon regeneration in the context of spinal cord injury [49]. Elevation of intracellular cAMP

has also been believed to promote axon regeneration by allowing neurons to overcome myelin inhibition through a transcription-dependent mechanism [67].

Modulating PTEN/mTOR Pathway

It was shown that PTEN deletion before axotomy ("pretreatment") promoted robust axon regeneration for the CST neurons (Fig. 21.2), which are otherwise highly refractory to regeneration [36]. A nongenetic method of suppressing PTEN with

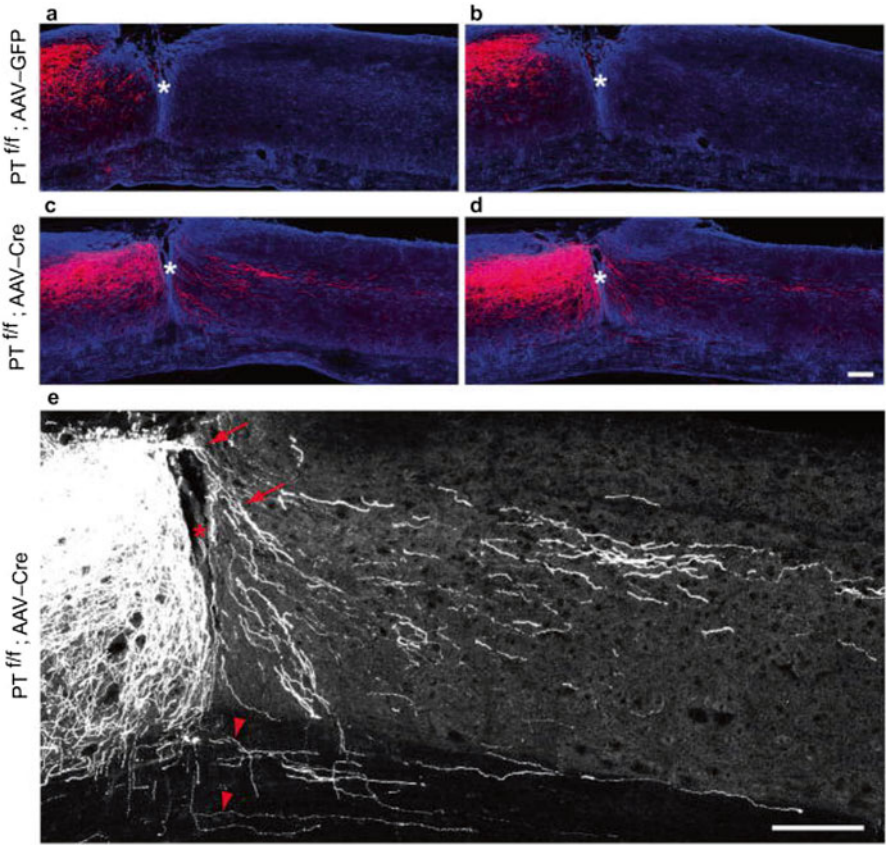

Fig. 21.2 PTEN deletion induces regeneration of corticospinal tract axons in a mouse model of spinal cord injury. In this model, T8 dorsal hemisection was performed in adult mice with floxed PTEN ($PT^{f/f}$) to completely transect all the corticospinal tract axons, which were traced by injecting biotinylated dextran amine (*red* axons in (**a**–**d**, *white* axons in **e**) in the cortex. Adeno-associated virus (AAV) mediated Cre expression was applied to delete PTEN in the corticospinal tract neurons in the cortex. In mice with AAV–GFP (control, **a**–**b**), no corticospinal tract axons grow across the lesion site (*asterisk* in **a**–**d**). Mice with AAV-Cre (**c**–**e**) injection show robust growth of corticospinal tract axons. Reactive astrocyte marker GFAP was shown in *blue* in **a**–**d**. Also see [36] (figure adopted with permission). Scale bars: 0.2 mm

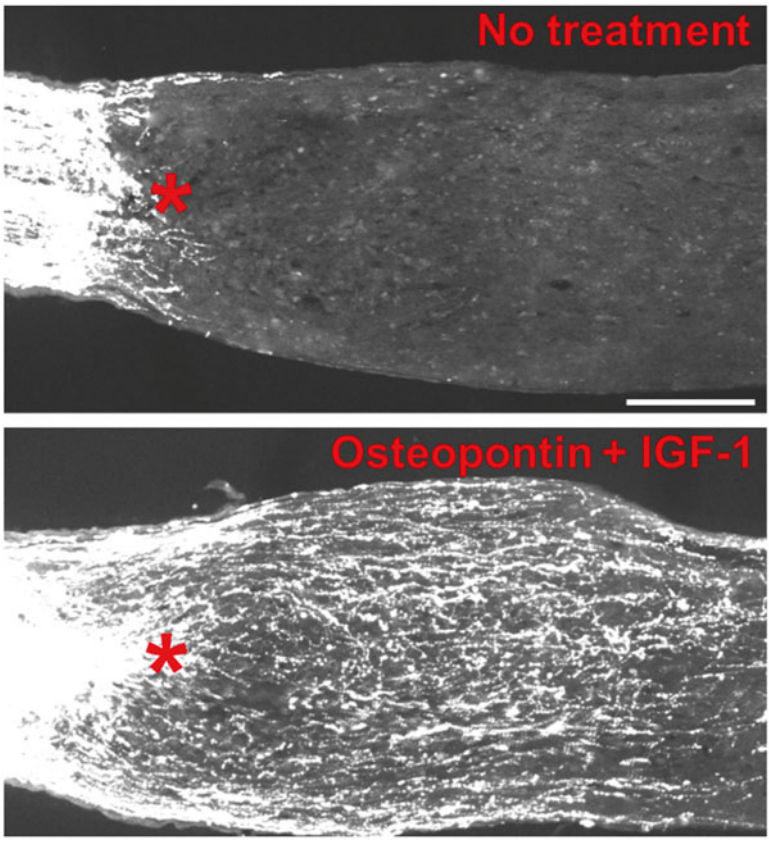

Fig. 21.3 Osteopontin promotes extensive axon regeneration in the mouse retinal ganglion cells when combined with the growth factor IGF-1. The mouse retinal ganglion cells, which are considered as part of the central nervous system, relay visual signal to the brain through their long-projecting axons in the optic nerve. An optic nerve crush (indicated by *asterisk*) transects all the axons and few of them regenerate as shown by anterograde tracing with cholera toxin beta subunit (*white*) 2 weeks after crush. AAV-mediated overexpression of osteopontin in the retinal ganglion cells induces extensive axon regeneration across the crush site when combined with exogenous application of recombinant IGF-1. This treatment of osteopontin plus IGF-1 is proposed to be a potentially safer "replacement" for PTEN suppression. Also see [73]. Scale bar: 0.1 mm

short-hairpin RNA also promoted CST axon regeneration and, when combined with delivery of salmon fibrin into the injury site, improved voluntary motor function after spinal cord injury [68, 69]. Deleting PTEN after axotomy ("post-treatment") was also effective in promoting axon regeneration [70]. Thus, PTEN has been shown to be an important target for promoting axon regeneration. However, PTEN is a tumor suppressor gene, deletion of which may increase the risk of certain cancers [71]. Carefully tuning down the PTEN activity ideally in a reversible manner is therefore recommended for therapy. Alternatively, decoding the molecular mechanism of PTEN deletion-induced axon regeneration and searching for molecular "replacement" for

PTEN deletion is preferable. Along this line, a recent study found that overexpression of osteopontin, a predominantly secreted phosphoprotein by many cells [72], increased mTOR activities in axotomized mouse RGCs and promoted RGC axon regeneration when combined with growth factors such as IGF-1 (Fig. 21.3; [73]). It was suggested that overexpression of osteopontin/IGF-1 may serve as one such "replacement" without the oncogenic complications associated with PTEN deletion [73].

Modulating SOCS3/STAT Pathway

SOCS3 is a negative regulator of the JAK–STAT pathway, which regulates cytokine signaling in the CNS [74, 75]. Deletion of SOCS3 triggered significant axon regeneration in the adult RGCs, especially when combined with exogenous application of CNTF [62]. Interestingly, SOCS3 deletion could work on top of PTEN deletion, together generating synergistic effect in axon regeneration [70].

Triggering Axon Regeneration by Inflammation

Surgical procedures in the eye such as nerve transplantation, lens injury, and intravitreous injection of proinflammatory agent Zymosan were found to trigger infiltration of inflammatory cells such as macrophages and neutrophils, which then activated axon regeneration in adult RGCs [76–79]. However, many cell types and effector molecules involved in the inflammatory responses may also exert negative effects in the CNS [80], so strategies of augmenting inflammation for better axon regeneration have to be carefully crafted to avoid such negative effects.

Perspectives

Much progress has been made in achieving successful axon regeneration by enhancing intrinsic growth ability in the adult CNS neurons. However, functional consequence of achieved regeneration after injury is far from certain. Even we assume optimal axon regeneration could be achieved and all the axotomized axons could regrow back to their target, we still need to ask: can they spontaneously reform functional synapses? Can they spontaneously integrate into the pre-existing circuits? Can system-level function be spontaneously restored? While the questions wait to be tested, it seems unlikely that regenerating axons is the only rate-limiting factor toward functional recovery, for example, after spinal cord injury. Much still needs to be done and a successful regenerative strategy is likely to require combinatorial modulations of both injured neurons and the environment they face.

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Chapter 22
Voltage-Gated Ion Channels as Molecular Targets for Pain

Gerald W. Zamponi, Chongyang Han, and Stephen G. Waxman

Abstract Pain signaling is critically dependent on voltage-gated ion channels that shape the action potential firing properties of peripheral afferents including pain-signaling dorsal root ganglion (DRG) neurons. Dysregulated expression of these critically important ion channels following nerve injury and in response to inflammation and gain-of-function changes in the channels due to mutations produce hyperexcitability which underlies pain. Thus, a major theme in translational research on pain has focused on the search for pharmacological modulators of ion channels, with an emphasis on development of modulators of peripheral channels that do not play major roles in the CNS or heart. This chapter summarizes recent advances on voltage-gated sodium, calcium, and potassium channels that are being explored as molecular targets for the treatment of pain.

Keywords Pain • Sodium channel • Calcium channel • Potassium channel • DRG

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Introduction

Nociception, the detection of noxious stimuli, plays a crucially important physiological role that provides protection from threatening external events and bodily injury. Activation of peripheral nerve endings by chemical, mechanical, or thermal stimuli triggers the production of action potentials that propagate centrally along primary afferent pain fibers that terminate within the spinal cord. Here, in the dorsal horn the primary afferents release neurotransmitters including glutamate and substance P, which activate second-order neurons that send the pain message to the brain. Voltage-gated ion channels are pivotal, in determining the action potential firing properties of peripheral afferents including pain-signaling dorsal root ganglion (DRG) neurons. Following nerve injury or in response to inflammation, the program of gene expression for ion channels changes within these cells, and these maladaptive changes in ion channel expression can produce hyperexcitability that results in chronic neuropathic or inflammatory pain (for a review, see [1, 2]). Gain-of-function mutations of ion channels expressed preferentially within DRG neurons can also produce pain [3–5]. Targeting the ion channels that shape the firing properties of afferent fibers, or that modulate the communication of primary afferents with second-order pain-signaling neurons, is thus a major focus in the search for new, more effective therapeutic interventions for neuropathic or inflammatory pain.

In this chapter, we expand on our recent review [6] and discuss voltage-gated ion channel targets that have garnered especially great interest as potential targets for pain therapy in humans. Although we discuss several types of ion channels that have been implicated in pain signaling, this chapter highlights several channel subtypes that have been validated by genetic studies in humans as potential therapeutic targets.

Sodium Channels as Molecular Targets for Pain

Voltage-gated sodium channels produce the inward transmembrane currents that underlie action potential firing within excitable cells, including pain-signaling neurons. It is thus not surprising that sodium channels have been considered as potential targets for pain pharmacotherapy. Nine different subtypes of voltage-gated sodium channels (named $Na_V1.1$ to $Na_V1.9$) are known to exist in mammals, all sharing a common overall structural motif, but with distinct amino acid sequences and different functional and pharmacological properties. The currently available sodium channel blockers that have been used in attempts to treat pain medications show limited clinical efficacy in most cases. This observation in the clinic probably reflects an important pharmacological feature of the existing blocking agents: the presently available medications block multiple subtypes of sodium channel in a nonspecific manner, inhibiting sodium channel subtypes that are expressed within brain and heart as well as those within pain-signaling neurons, so that their clinical

efficacy is constrained by dose-limiting central side effects that include diplopia, ataxia, confusion, and somnolence.

"Peripheral" Sodium Channels

Given the above, it is not surprising that substantial effort has been devoted to the identification of sodium channel subtypes that play major roles in electrogenesis within pain-signaling peripheral neurons, but are not essential for function of CNS neurons or cardiac myocytes. Three sodium channel subtypes ($Na_V1.7$, $Na_V1.8$, and $Na_V1.9$) appear to fulfill these criteria, and a fourth ($Na_V1.3$) also falls into this category because it is upregulated within DRG neurons following nerve injury. Three of these sodium channel subtypes, $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$, have been validated as major players in human pain by genetic studies; this may be especially important because studies in animal models have proven to have limited predictive value in terms of human therapeutic responses.

$Na_V1.7$

The $Na_V1.7$ voltage-gated sodium channel, originally called PN1 and hNE, is preferentially expressed in peripheral neurons, including dorsal root ganglion (DRG), trigeminal and nodose ganglion, and sympathetic ganglion neurons [7, 8]. $Na_V1.7$ is expressed along the entire length of the axons of DRG neurons, from their peripheral arborizations to their central terminations within the dorsal horn [3].

$Na_V1.7$ is characterized biophysically by slow closed-state inactivation and relatively hyperpolarized voltage dependence; as a result, this channel is activated by slow, small depolarizations close to resting potential, so that it sets the gain on nociceptors [3]. Knockout of $Na_V1.7$ within mouse DRG neurons that express $Na_V1.8$ attenuates inflammation-induced pain and thermal hyperalgesia induced by burn injury [9, 10]. Consistent with this, inflammation within the projection fields of DRG neurons triggers an upregulation of $Na_V1.7$ expression within these cells [3]. Also providing a link to pain, abnormal accumulations of $Na_V1.7$ are known to be present within the injured blindly ending axon tips which act as sites of inappropriate ectopic impulse generation within experimental and human neuromas [11]. Minett et al. [12] have reported that knockout of $Na_V1.7$ in both DRG and sympathetic neurons is required for a phenotype in which neuropathic pain develops after nerve injury, and have interpreted this observation as suggesting that, at least in this mouse model, $Na_V1.7$ expression in sympathetic neurons is required for establishment of neuropathic pain.

Validation of $Na_V1.7$ as a pain target *in humans* was provided by genetic studies and functional profiling of mutant $Na_V1.7$ channels, in subjects with rare hereditary pain disorders. Yang et al. [13] used linkage analysis to study two families with the

autosomal dominant disorder inherited erythromelalgia (IEM), in which patients experience severe burning pain that is triggered by mildly warm stimuli, and demonstrated a link of missense mutations in *SCN9A*, the gene encoding $Na_V1.7$. Cummins et al. showed that these hyperpolarize activation of $Na_V1.7$ (making it easier to activate these channels) and increase the channels' response to small depolarizing stimuli [14]. At the channel level, the mutations are thus gain of function. Current-clamp studies [3] subsequently permitted an examination of the effect of these mutations on cellular function, and showed that these mutations produce gain-of-function changes in DRG neurons, lowering their threshold via a hyperpolarizing shift in activation and increasing the frequency at which they fire in response to graded suprathreshold stimuli (Fig. 22.1).

A different group of gain-of-function mutations of $Na_V1.7$, which impair channel inactivation, was identified several years later in patients with another distinct hereditary pain disorder, Paroxysmal Extreme Pain Disorder (PEPD) characterized clinically by severe rectal pain triggered by lower body stimulation, later in life migrating to peri-orbital and peri-mandibular regions [15]. Thus, two rare genetic disorders, both characterized by severe pain, were linked to gain-of-function mutations in $Na_V1.7$.

Providing additional validation of a role of $Na_V1.7$ in human pain signaling, loss-of-function mutations of $Na_V1.7$ have also been identified in families with an autosomal recessive syndrome of channelopathy-associated insensitivity to pain (this disorder is distinct from the syndrome of congenital insensitivity to pain that occurs due to defects in NGF signaling). As a result of truncation and similar mutations, humans with $Na_V1.7$ channelopathy-associated insensitivity to pain do not produce functional $Na_V1.7$ channels, and display painless bone fractures, burns, tooth extractions, and childbirth [16–18]. It is not fully known, at this time, whether $Na_V1.7$ has a pivotal role in pain signal transmission at a singular site within primary nociceptive neurons (e.g., within peripheral nerve endings, sensory axon trunks, or dorsal horn preterminal or terminal axons). Whether there are secondary changes in pain circuitry within the CNS in subjects in whom, due to lack of functional $Na_V1.7$ channels, there is a relative lack of afferent activity in primary nociceptors during critical periods of development, has not yet been determined.

Fig. 22.1 (continued) Comparison of steady-state activation and fast inactivation for wild-type and F1449V channels. F1449V mutation causes the activation shift to the hyperpolarizing direction by 7.6 mV and the fast-inactivation shift to the depolarizing direction by 4.3 mV. (**d**) Comparison of steady-state slow inactivation for wild-type and F1449V channels. F1449V mutation shifts the slow inactivation in the hyperpolarizing direction. (**e**–**h**) Current-clamp analysis of DRG neurons expressing wild-type and F1449V mutant channels. (**e** and **f**) Representative traces from DRG neurons expressing wild-type $Na_V1.7$ (**e**) or $Na_V1.7$ with the F1449V mutation (**f**). The traces show that neurons expressing the F1449V mutant channels have a reduced current threshold for action potential generation. (**g** and **h**) A DRG neuron expressing wild-type channels (**g**) generates less number of action potential spikes in response to a 950-ms stimulation of 150-pA than does the DRG neuron expressing the F1449V mutant channels (**h**) (same cells as in **e** and **f**). All parts of the figure were modified, with permission, from Dib-Hajj et al. [119] © (2005) Oxford University Press

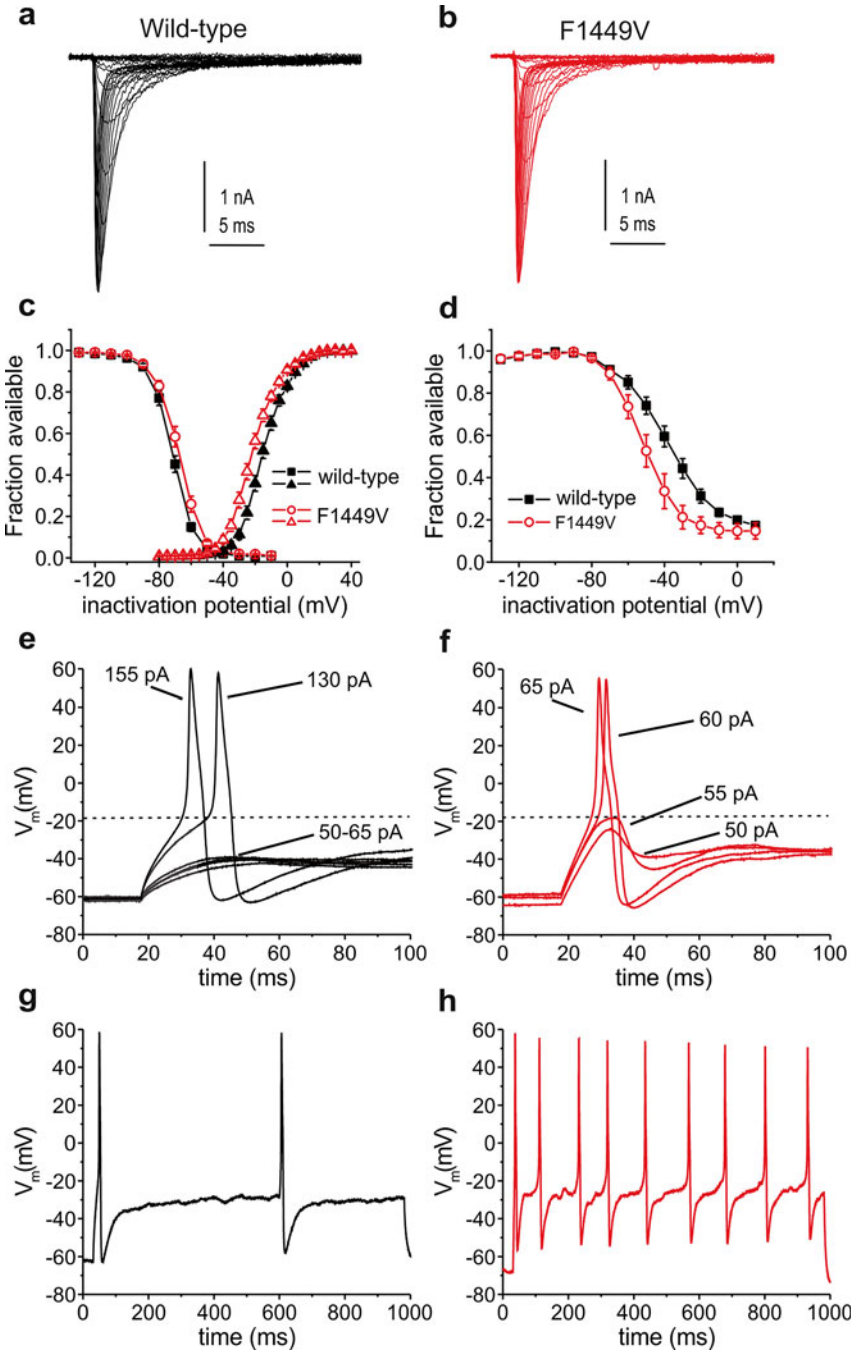

Fig. 22.1 The inherited erythromelalgia (IEM)-related $Na_V1.7$ mutation F1449V causes gain-of-function alterations in $Na_V1.7$ and makes DRG neurons hyperexcitable. (**a**–**d**) Voltage-clamp analysis of wild-type and F1449V channels in HEK293 cells. (**a** and **b**) Current traces recorded from representative HEK293 cells expressing either wild-type (**a**) or F1449V (**b**) channels. (**c**)

Recent studies have extended the link between $Na_V1.7$ and human pain to more common disorders. Estacion et al. described a polymorphism (R1150W) in the gene encoding Nav1.7 that is present in nearly 30 % of some control populations and demonstrated that this single amino acid substitution produces a moderate degree of hyperexcitability of DRG neurons, suggesting that it might increase pain sensitivity or susceptibility to pain after injury [19]. Consistent with this suggestion, genome-wide association studies have demonstrated an association of the minor allele with increased pain scores in osteoarthritis, compressive radiculopathies, and traumatic limb amputation [20].

Faber et al. extended the study of $Na_V1.7$ to small-fiber peripheral neuropathy, a relatively common disorder in which patients experience severe pain. They demonstrated gain-of-function variants of $Na_V1.7$, which substitute single amino acids within the intracellular loops and linkers of the channel protein, in nearly 30 % of patients with this disorder [21]. Consistent with the location of the amino acid substitutions within the intracellular loops and linkers rather than within membrane-spanning segments of the channels, these mutations tend to produce relatively subtle gain-of-function abnormalities in biophysical properties of the $Na_V1.7$ channel, including impairment of slow inactivation or impairment of both fast and slow inactivation. In some cases these mutations also enhance activation and enhance the persistent (non-inactivating) current produced by the channel. At the cellular level, these mutations lower action potential threshold and produce higher-than-normal firing frequencies and aberrant spontaneous firing in DRG neurons. The changes in DRG excitability provide a basis for the evoked and spontaneous pain reported by these patients with painful neuropathy [21]. An example is shown in Fig. 22.2.

Largely on the basis of these observations, $Na_V1.7$ has evoked substantial interest as a human pain target, and selective blockers of this channel are being developed

Fig. 22.2 (continued) inactivation for wild-type and I720K channels. I720K does not alter activation or fast inactivation. (**d**) Comparison of steady-state slow-inactivation curves between wild-type and I720K mutant channels. I720K mutation impaired steady-state slow inactivation by 8.8 mV. * $p<0.05$. (**e**–**h**) Current-clamp analysis of DRG neurons expressing wild-type and I720K mutant channels. (**e**) Compared with wild-type (-55.8 ± 1.7 mV, $n=26$), I720K mutant channels (-48.7 ± 1.9 mV, $n=29$) significantly depolarized the resting membrane potential by 7.1 mV. $^*p<0.05$. (**f**) Current threshold of DRG neurons transfected with I720K (134 ± 30 pA, $n=29$) was significantly smaller than wild-type channels (237 ± 28 pA, $n=26$). $^*p<0.05$. (**g**) DRG neurons expressing I720K mutant channels demonstrated to fire at significantly higher frequencies than DRG neurons expressing wild-type channels across a range of current injections from 100 to 600 pA; $^*p<0.05$. (**h**) I720K mutation displayed a trend to increase the proportion of spontaneously firing neurons. The bar graph on the left of the panel shows that the proportion of spontaneous firing cells for DRG neurons expressing I720K (24 %, 9 of 38) was increased by 17 % compared with that for DRG neurons expressing wild-type channels (7 %, 2 of 28; $p=0.075$). A10-s long representative spontaneous firing trace recorded from a DRG neuron expressing I720K was shown on the right of the panel. All parts of the figure were modified, with permission, from Faber et al. [21]

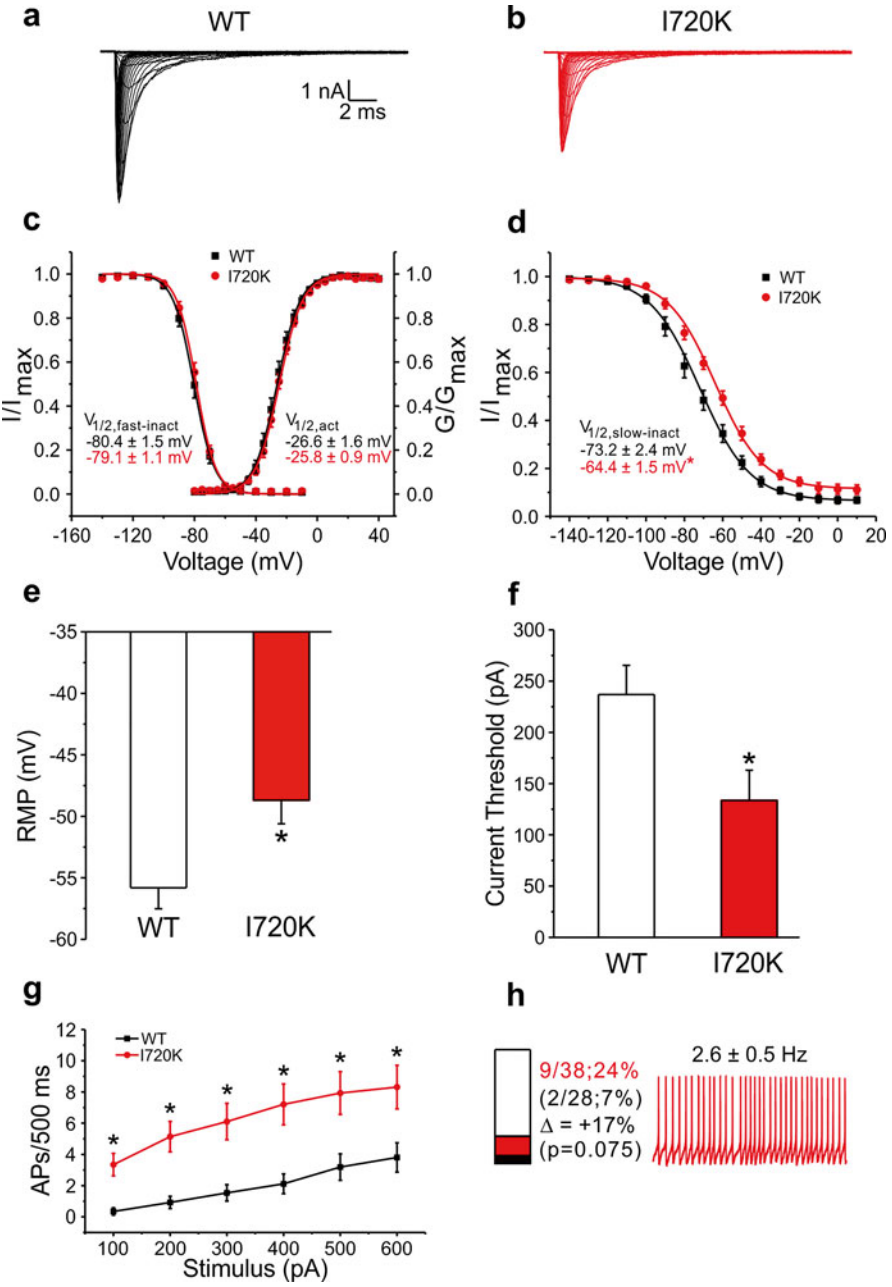

Fig. 22.2 The small-fiber neuropathy (SFN)-related $Na_V1.7$ mutation I720K impairs slow inactivation and renders DRG neurons hyperexcitable. (**a**–**d**) Voltage-clamp analysis of wild-type and I720K channels in HEK293 cells. (**a** and **b**) Representative current traces recorded from HEK293 cells expressing wild-type (**a**) or I720K (**b**). (**c**) Comparison of activation and steady-state fast

as potential pain therapeutics. An agent that blocks $Na_V1.7$ (although not in a specific manner) has been reported to attenuate pain in a small number of patients with inherited erythromelalgia [22], suggesting that target engagement may be of therapeutic value. Clinical studies have begun with several isoform-specific $Na_V1.7$ blockers.

There are several caveats to the strategy of subtype-specific $Na_V1.7$ blockade. First, as a result of the presence of $Na_V1.7$ in sensory neurons other than pain-signaling neurons, such as olfactory sensory neurons, the possibility exists that there may be clinically significant sensory side effects of block; anosmia has been noted in patients with channelopathy-associated insensitivity to pain [23]. Second, while $Na_V1.7$ is enriched in peripheral neurons, it has been shown in rodents to be present at detectable levels in some CNS neurons, e.g., hypothalamic supraoptic neurons [24]. There will thus be a need for careful assessment of potential adverse effects in this regard. Finally, when $Na_V1.7$ blockers are studied in the clinic, it would be ideal to titrate the therapeutic effect so that pathological pain is reduced, but normal nociceptive pain not totally eliminated, and this may require particular attention to dosing.

Recent studies also raise the possibility of targeting $Na_V1.7$ in a personalized, genomically guided manner. Yang et al. [25] built upon the observation of a rare family with inherited erythromelalgia with a $Na_V1.7$ mutation (V400M) that responds to treatment with carbamazepine. Based upon the observation that the V400M mutation makes the channel sensitive to carbamazepine [26], Yang et al. [25] used atomic-level structural modeling and thermodynamic analysis, incorporating the carbamazepine-responsive mutation as a "seed," and predicted the carbamazepine responsiveness of other $Na_V1.7$ variants [25]. These results support the idea that genomically guided pain pharmacotherapy may be an achievable goal.

$Na_V1.8$

Although less data is available from human studies, sodium channel $Na_V1.8$, originally called SNS (Sensory Neuron Specific), has also been firmly linked to human pain. $Na_V1.8$ plays a crucial functional role in DRG neurons and has recently been linked to painful neuropathies in humans. Notably, $Na_V1.8$ is expressed specifically within DRG neurons and their axons as well as trigeminal and nodose ganglion neurons in the normal nervous system. $Na_V1.8$ differs from other Na channel subtypes in that its voltage dependence is relatively depolarized. As a result, this sodium channel subtype is relatively resistant to inactivation when a neuron is depolarized [27]. Also contributing to its functional role in DRG neurons, $Na_V1.8$ reprimes rapidly (recovers rapidly from inactivation) and produces the majority of the inward current underlying the action potential upstroke, so that it supports repetitive firing in DRG neurons when these cells are depolarized [28]. Inflammatory mediators increase the $Na_V1.8$ current via p38-mediated phosphorylation [29, 30]. Knockout [31] and knockdown studies in rodents using small-molecule blockers [32] and

antisense oligonucleotides [33] provide additional evidence for a contribution of $Na_V1.8$ to inflammatory and possibly neuropathic pain.

A link of $Na_V1.8$ to pain in humans has been provided by recent studies on patients with painful peripheral neuropathy. These studies demonstrated gain-of-function mutations in $Na_V1.8$ in about 5 % of patients with painful peripheral neuropathy who do not carry $Na_V1.7$ mutations [4]. At the cellular level, these gain-of-function $Na_V1.8$ mutations produce hyperexcitability and aberrant spontaneous firing in DRG neurons, again providing a firm pathophysiological basis for evoked and spontaneous pain. Blockers of $Na_V1.8$ are under development.

$Na_V1.9$

The $Na_V1.9$ sodium channel, which was initially called NaN (Na channel, Nociceptive), is specifically expressed within peripheral sensory neurons such as DRG and trigeminal ganglion neurons as well as myenteric plexus neurons, particularly nociceptors. $Na_V1.9$ is unique in producing a non-inactivating current that is activated at relatively hyperpolarized potentials close to resting potential [34]. Because of its slow kinetics, $Na_V1.9$ does not contribute substantially to the rapid upstroke of the action potential. The functional role of $Na_V1.9$ is to depolarize these cells so that it prolongs and enhances small depolarizations, thus increasing excitability [34–37]. An increase in the $Na_V1.9$ current is triggered by inflammatory mediators [29, 38], suggesting a contribution of $Na_V1.9$ in inflammatory pain signaling. Consistent with this, $Na_V1.9$ knockout mice display attenuated inflammatory pain behavior [39, 40]. A strong upregulation of $Na_V1.9$ within DRG neurons has been observed in a rat model of diabetic neuropathy, suggesting a contribution to diabetic neuropathic pain [41].

Several studies have recently shown that $Na_V1.9$ plays a role in human pain. Zhang et al. [42] reported two point mutations in $Na_V1.9$, which segregated with an autosomal dominant phenotype of episodic pain in affected subjects from two multi-generation families. Voltage clamp showed that these mutations produce an increase in the current density of $Na_V1.9$, and current-clamp recordings showed that they produce increased excitability of DRG neurons. Huang et al. [5] described 11 missense variants of $Na_V1.9$ within a series of 344 patients with painful, predominantly small-fiber neuropathy who did not carry mutations of $Na_V1.7$ or $Na_V1.8$. Four of these mutations substituted amino acids in conserved, membrane-spanning regions of the channel. Two of these mutations have thus far been shown to confer gain-of-function changes at the voltage-clamp and current-clamp level.

Thus far, low levels of $Na_V1.9$ current within heterologous expression systems have limited the development of $Na_V1.9$-specific blockers. The recent link of $Na_V1.9$ to human pain will undoubtedly trigger increased interest in this channel, and it is likely that more effective expression systems will be developed, permitting high- or at least medium-throughput screening of candidate blockers.

$Na_V1.3$

The $Na_V1.3$ subtype of the voltage-gated sodium channel has been of special interest in pain research because the level of expression of this channel, which is not detectable within the adult rodent nervous system, increases within DRG neurons following peripheral nerve injury, probably as a result of deprivation of access to a peripheral pool of trophic factors including NGF [43, 44]. $Na_V1.3$ produces a persistent current and responds to small ramp-like depolarizations close to resting potential, poising it to amplify small inputs. $Na_V1.3$ also recovers rapidly from inactivation, thereby supporting repetitive firing [45]. Interest in $Na_V1.3$ as a potential pain target was supported by reports of increased $Na_V1.3$ expression within second- and third-order neurons along the pain-signaling pathway within dorsal horn and thalamus, and by the observation of rescue of relatively normal excitability in these neurons and of attenuation of pain following $Na_V1.3$ knockdown with antisense in rat models of peripheral nerve injury and spinal cord injury [46]. Consideration of $Na_V1.3$ as a pain target waned, however, as a result of reports of lack of a pain phenotype in at least some knockouts and failure to observe attenuation of pain behavior in peripheral nerve injury models with a different antisense construct [47, 48]. Recently, however, interest in $Na_V1.3$ as a pain target has increased. Samad et al. [49] used AAV delivery of $Na_V1.3$ shRNA for specific knockdown of $Na_V1.3$ within a rodent neuropathic pain model, and observed a statistically significant attenuation of pain behavior as a result of knockdown. Further studies of $Na_V1.3$ as a potential therapeutic target for pain are under way.

Voltage-Gated Calcium Channels

Voltage-gated calcium channels are a key source of calcium in response to membrane depolarization. Neurons express nine types of voltage-gated calcium channels which fall into three major families—Ca_V1, Ca_V2, and Ca_V3 (reviewed in [1, 50]). The Ca_V1 family represents L-type calcium channels [50]. While these channels are interesting and contribute to many physiological and pathophysiological processes, their roles in pain signaling are thought to be limited [1]. The Ca_V2 family includes N-, P/Q-, and R-type channels, which share the physiological function of controlling the release of neurotransmitters from presynaptic nerve terminals [50, 51]. N-type calcium channels are of particular relevance to pain signaling, because afferent nerve terminals in the dorsal horn of the spinal cord rely heavily on this calcium channel subtype for the release of glutamate, substance P, and CGRP [1]. The Ca_V3 family represents three different T-type calcium channel isoforms [52], with $Ca_V3.2$ being of particular importance for regulating the excitability of afferent neurons. For the purpose of this chapter, we shall focus predominantly on N- and T-types.

The Ca_V2 family belongs to the class of high voltage activated channels which are heteromultimers that comprise a pore-forming $Ca_V\alpha1$ subunit, a cytoplasmic

$Ca_V\beta$ subunit, and a largely extracellular $Ca_V\alpha2\delta$ subunit [53]. Four different genes encode different types of these two subunits, and their primary roles are to enhance channel trafficking to the plasma membrane plus a regulation of channel gating properties [53]. T-type calcium channel appears to lack these ancillary subunits. Both N-type and T-type channel expression are aberrantly enhanced in afferent fibers under chronic pain conditions, and both channel subtypes are considered potential pharmacological targets for the treatment of pain. Curiously, unlike in the case of sodium channels, there are no reported calcium channel mutations that have been linked to persistent pain in humans.

N-type Calcium Channels

N-type calcium channels are tightly coupled to the neurotransmitter release machinery in presynaptic nerve terminals [54], such that calcium entry via this channel results in rapid exocytosis [55]. Hence, inhibition of these channels results in a reduction of neurotransmitter release. This is of particular relevance to pain signaling as the communication between primary afferent neurons and second-order neurons that project to the brain depends critically on N-type calcium channels [1]. The pore-forming $Ca_V2.2$ subunit of the N-type calcium channel complex has been shown to undergo alternate splicing at several loci, including the exon 37 [56, 57]. It has been shown that channels that contain exon 37a are preferentially expressed in small nociceptive neurons [56] and that they are the primary splice isoform responsible for transmission of pain signals under inflammatory and neuropathic conditions [58]. Hence, from a therapeutic standpoint, the ability to selectively target exon37a containing channels would be beneficial, albeit technically challenging.

N-type calcium channel function is potently regulated by a wide array of G protein coupled receptors (GPCRs) [59]. Upon receptor activation, the $G\beta\gamma$ subunit complex physically associates with the channel to stabilize the close conformation, thereby reducing whole cell calcium current and thus neurotransmitter release [60, 61] (Fig. 22.3). This is exemplified by the actions of μ-opioid receptors, the key target for morphine, a widely used clinical pain drug [62]. All other members of the opioid receptor family including the nociception receptor have been shown to mediate analgesia when delivered intrathecally to rodents [61, 63–65]. Along these lines, GABA–B receptor activation reduces neurotransmitter release in certain dorsal horn synapses to inhibit the transmission of pain signals [66]. Opioids such as morphine are highly effective, but also prone to side effects such as constipation and respiratory depression, and are subject to the development of tolerance [67, 68]. Although morphine remains the drug of choice for a range of severe pain conditions, there is a need to develop N-type channel antagonists that are not subject to these limitations. This includes both agonists of other types of GPCRs, and direct inhibitors of channel function.

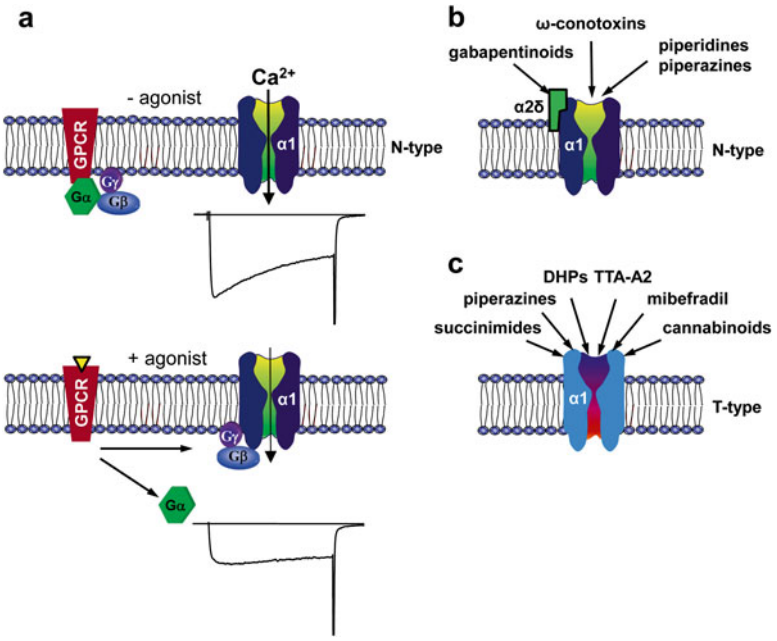

Fig. 22.3 Inhibition of N-type and T-type calcium channels for pain therapeutics. (**a**) *Top*: In the absence of agonist, G protein coupled receptors (GPCRs) such as opioid, nociceptin, and GABA-B receptors are inactive, resulting in normal calcium influx via N-type calcium channels and large whole cell N-type currents (see current tracing). *Bottom*: Activation of these receptors through agonist binding (indicated by *yellow triangle*) results in the association of the Gβγ dimer with the pore-forming $Ca_V\alpha1$ subunit of the N-type calcium channel. This reduced calcium influx, and thus the amplitude of the whole cell current (see current trace), in addition to slowing the macroscopic time course of inactivation. Ancillary calcium channel $Ca_V\beta$ and $Ca_V\alpha2\delta$ subunits have been omitted for clarity. (**b**) Classes of N-type channel blocking drug molecules that are in clinical use, or in preclinical development for the treatment of pain. Gabapentinoids interact with the ancillary $Ca_V\alpha\delta$ subunit (depicted in *green*) to reduce cell surface trafficking of the N-type calcium channel complex. Peptide toxins such as ω-conotoxin MVIIA physically block the pore of the channel. Piperazines and piperidines are scaffolds for state-dependent inhibition of N-type channels. (**c**) Classes of T-type channel blocking drug molecules that are in preclinical development for the treatment of pain. Most of these drug classes mediate state-dependent inhibition of T-type channels

Therapeutically useful direct blockers of N-type calcium channels come in two major classes—peptide blockers and small organic molecule inhibitors (Fig. 22.3). A number of highly selective peptide blockers of N-type channels have been isolated from the venoms of predatory marine snails, including *Conus geographus*, *Conus magus*, and *Conus catus* [69, 70]. This includes ω-conotoxin MVIIA, as small cysteine-rich peptide that virtually irreversibly blocks the pore of the channel [71]. This toxin can be synthesized in vitro and has been shown to mediate potent analgesia when delivered intrathecally [72]. It has been approved for human use in a subset of cancer patients under the trade name Prialt (formerly known as ziconotide) [73, 74]. However, it has a relatively narrow therapeutic window [75], and if not dosed correctly, can result in side effects that include memory loss, auto-

nomic dysfunction, and unruly behavior [76, 77]. A second-generation cone peptide from *Conus catus* ω-conotoxin CVID has a wider therapeutic window and was explored in phase II clinical trials in Australia [70]. Other related derivatives of this peptide are in preclinical development. While these toxins provide good proof of concept for targeting N-type channels to block pain, the fact that they do not cross the blood–brain barrier and the reported side effects limit their usefulness as therapeutics.

An alternative strategy is the use of small organic state-dependent N-type channel inhibitors. Numerous pharmacophores for such compounds have been identified in high-throughput screening assays, in many cases with a piperidine or piperazine core [78–81]. In animal studies, some of these compounds have shown remarkable efficacy [79]. However, one of the most promising preclinical compounds (Z-160) recently failed phase II clinical trials. Whether other small organic N-type channel blockers that are currently under preclinical development will meet a similar fate remains to be seen.

An unconventional strategy for targeting N-type calcium channels is a novel peptide toxin termed Vc1.1. This α-conotoxin inhibits N-type channels via GABA-B receptors and mediates analgesia in rodent models of pain [66, 82, 83]. Remarkably, this toxin is orally bioavailable and it will be interesting to see if it is active in humans.

As noted earlier, N-type calcium channels are part of a heteromeric assembly that includes a $Ca_V\alpha2\delta$ subunit that is very important for proper trafficking of the channel to the plasma membrane [84]. Hence, interfering with the expression or the function of this subunit has the propensity to reduce N-type channel density. Gabapentin and pregabalin (Lyrica) are compounds that both act at a specific amino acid residue of the $Ca_V\alpha2\delta$ subunit [85]. Both of these compounds are effective in neuropathic pain in human patients, possibly because they might regulate the membrane expression of N-type calcium channels in dorsal horn synapses by virtue of interfering with the function of $Ca_V\alpha2\delta$ [84], and thus synaptic function [86]. However, while the molecular target for gabapentinoids has been clearly identified [87], there is still no concrete evidence as to whether its analgesic actions are indeed mediated via altered N-type channel currents.

Altogether, although N-type channels have been validated as therapeutic targets for treating pain in humans, the efforts toward development of orally bioavailable small organic inhibitors have not yet come to fruition.

T-type Calcium Channels

In the context of afferent pain signaling, T-type calcium channels play two major roles. Because of the relatively hyperpolarized voltage dependences of activation and inactivation, and their ability to support window currents, these channels are well suited toward regulating neuronal excitability [88, 89]. In addition, it has been reported that T-type calcium channels contribute to spontaneous neurotransmitter release in dorsal horn synapses [90]. In both cases, enhanced T-type calcium

channel expression or activity is expected to mediate proalgesic effects. In response to peripheral nerve injury, functional T-type channel expression in primary afferent fibers is enhanced [91]. Along these lines, inflammation of the colon triggers an upregulation of T-type channels in sensory neurons that innervate the inflamed tissue [92]. Finally, in mouse models of diabetic neuropathy there is an increase in T-type channel expression in sensory neurons [93]. Hence, there is a common thread in that injuries that trigger the activation of pain fibers lead to a persistent upregulation of T-type channels. It has been shown that $Ca_V3.2$ is the major Ca_V3 isoform that is responsible for T-type currents in dorsal root ganglion neurons [94]. Antisense depletion of these channels in these neurons protects rodents from both neuropathic and inflammatory pain, thus underscoring the importance of $Ca_V3.2$ channels in this process [94]. Mice lacking $Ca_V3.2$ altogether display some protection from inflammatory pain; however [95], there is likely compensation from other channels in these animals. Indeed, acute block of these channels by intrathecal delivery of T-type channel inhibitors mediates potent analgesia [96, 97]. This includes compounds such as ethosuximide, the relatively new compound TTA-A2 [98], the cannabinoid ligand anandamide [99, 100], and several other derivatives of cannabinoids [101, 102] (Fig. 22.3). Many of these compounds are ineffective in $Ca_V3.2$ null mice [103], indicating that their analgesic actions are mediated by inhibition of $Ca_V3.2$ channels, rather than other molecular targets.

It is interesting to note that T-type calcium channels share some sequence homology with voltage-gated sodium channels. Specifically, a number of amino acid residues that form the local anesthetic receptor site in sodium channels are conserved in $Ca_V3.2$ calcium channels, and consequently, several blockers that act on sodium channels also block T-types, albeit with somewhat lower affinity [104]. Nonetheless, these observations suggest the possibility of using homology modeling toward the rational design of new T-type calcium channel antagonists. It should also be noted that there are a number of structurally very different pharmacophores that are able to interact with T-type channels, including piperazine derivatives [105], as well as certain dihydropyridines [106, 107]. A number of pharmaceutical companies are actively pursuing T-type calcium channel blockers, including Zalicus who are currently conducting clinical trials with their T-type inhibitor Z944.

Altogether, T-type calcium channels appear to be suitable targets for the development of novel pain therapeutics, but to date, there is no known T-type channel blocker approved for humans specifically for treating pain.

Voltage-Gated Potassium Channels

Potassium channels regulate the resting membrane potential of neurons and mediate the downstroke of the action potential. The mammalian genome encodes more than 70 different types of potassium channel α subunits which can form homo- and heterotetramers [108]. In addition, certain types of potassium channels co-assemble

with ancillary β subunits and accessory proteins such as KChIPs and DPPs [109, 110]. The potassium channel family is diverse and includes channels that are activated by voltage, ions such as calcium and sodium, and leak channels such as inward rectifiers, each with a characteristic transmembrane topology. It is becoming increasingly evident that potassium channels play important roles in the excitability of primary afferent fibers and second-order neurons that project to higher brain centers. The archetype potassium channel associated with pain signaling is the G protein coupled inwardly rectifying potassium (GIRK) channel whose activation by opioid and GABA-B receptors produces analgesia [111]. Below we will focus on members of the voltage-gated, ion-gated, and two pore families and their emerging roles in pain signaling.

Voltage-gated potassium (K_V) channels are important regulators of neuronal firing frequency and output gain. Primary afferent fibers contain several different types of K_V channels from multiple K_V families [112–115]. The expression of specific types of K_V channels can be altered under various pain conditions, and this plasticity may ultimately contribute to hyperexcitability of pain fibers. Notably, patients with autoantibodies against various K_V channels may present with thermal hyperalgesia [116]. In a model of diabetic neuropathic pain, I_A currents are downregulated at the mRNA levels, whereas sustained K_V currents appear unaltered [112]. This effect is mediated, at least in part, by elevation of BDNF (perhaps released from activated microglia). Consequently, antibodies to BDNF reportedly prevent the downregulation of I_A currents in diabetic animals. These data indicate that the expression of K_V subunits can be aberrantly regulated by growth factors and that interfering with this process may be a suitable strategy for combatting the development of pain hyperexcitability under conditions such as diabetes. Downregulation of K_V channels has also been reported in response to direct nerve injury. For example, in axotomized neurons, K_V9.1 channels are downregulated. Interestingly, this particular type of potassium channel cannot produce functional currents on its own, but instead co-assembles with K_V2.1 subunits [114]. Therefore, it is likely that a decrease in K_V9.1 results in a corresponding decrease in K_V9.1/K_V2.1 complexes, which then results in neuronal hyperexcitability of DRG neurons. Indeed, the reduction of K_V9.1 expression is paralleled by a development of neuropathic pain [114]. In response to nerve injury, there also appears to be a reduction in the expression of K_V1.2 channels [117]. This reduction is due to a somewhat unusual mechanism that involved the aberrant expression of a small RNA sequence that is complementary to K_V1.2. This in turn depresses K_V1.2 expression to trigger enhanced afferent fiber excitability. Interestingly, the expression of this RNA is mediated by myeloid zinc finger protein 1, raising the possibility that drugs that target this protein could perhaps be used as analgesics.

Members of the K_V7 family also appear to show alterations in expression under chronic pain conditions. For example, in a rat model of bone cancer there is a reduced expression of K_V7.2 and K_V7.3 subunits. This in turn leads to a reduced whole cell M current in DRG neurons [115] and consequently an increase in excitability. The resulting increase in mechanical allodynia and thermal hypersensitivity

can be alleviated by administration of the K_V7 opener retigabine [115]. Similar benefits have been observed in models of inflammatory joint pain [118]. Hence, openers of voltage-gated potassium channels may be a viable therapeutic approach for combatting pain.

Horizons and Prospects

Many types of voltage-gated ion channels have been associated with the development of chronic pain. As we have outlined above, voltage-gated sodium, potassium, and calcium channels play important roles in pain signaling, and the expression of these channels is altered in response to nerve injury or peripheral inflammation. Consequently, it does not seem unreasonable to suggest that pharmacological manipulation of channel activity to compensate for these changes may be a suitable therapeutic approach toward pain—in the case of potassium channels, this would involve a stimulation of channel activity, whereas in the case of sodium and calcium channels, blockers would be required. Remarkably, very few modulators of these channel types have thus far been approved for use in the clinic, despite promising preclinical data. Sodium channels take a special place among these channel families, as genetic mutations of these channels have been linked to either persistent pain, or persistent loss of pain syndromes. Why similar mutations have not been described in calcium and potassium channels is a mystery. With that being said, the linkage between amino acid substitutions and pharmacological properties of channels may in the future provide a basis for personalized, pharmacogenomically guided treatment of painful conditions [25].

Much remains to be learned about ion channels and pain, and the leap from laboratory to clinic will be challenging. Nonetheless, our understanding of these molecules and their critically important roles in pain suggest that we can be optimistic about the development of new approaches that will target them in a specific manner in the clinic. Hopefully this will lead to the development of new and more effective treatments for pain.

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Part IV
Activity-Dependent Plasticity and Neurorehabilitation

Chapter 23
Rehabilitation-Dependent Neural Plasticity After Spinal Cord Injury

Lea Awai, Volker Dietz, and Armin Curt

Abstract Complex movements are programmed in the central nervous system (CNS) and adapted by proprioceptive feedback. The selection of and interaction between different sources of afferent input is task dependent. Simple stretch reflexes are thought to be involved primarily in the control of focal movement. For more complex motor behaviors such as locomotion, afferent input related to load and hip-joint position probably has an important role in the proprioceptive contribution to the activation pattern of the leg muscles. Advances in our understanding of movement control allow us to define more precisely the requirements for the rehabilitation of patients with movement disorders. Accordingly, acknowledging the discrepancy between spasticity as assessed by clinical bedside testing and spasticity as presented in movement disorders affecting gait is essential to appreciate the true impact of spasticity. Central motor lesions are associated with a loss of supraspinal drive and defective use of afferent input. These changes lead to paresis and maladaptation of the movement pattern. Secondary changes in mechanical muscle fiber and collagen tissue result in spastic muscle tone, which in part compensates for paresis and allows functional movements on a simpler level of organization. The respective contributions to an aberrant gait pattern are complex and the resolution benefits from applying detailed kinematic movement analyses complementary to clinical measures to reveal changes in motor control. The distinct capacity of subjects with an incomplete spinal cord injury (iSCI) to remain able to modulate time–distance parameters but revealing complex impairments of intralimb coordination and the dissimilar responsiveness to rehabilitative interventions reveal distinct domains of neural control of walking. More sensitive outcome measures will be essential to uncover the respective contributions of restitution (i.e., repair of damaged neural structures) and mechanisms attributable to adaptation and compensatory movement strategies to rehabilitation-dependent functional improvements.

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Abbreviations

6minWT	6-min walk test
10MWT	10-m walk test
AIS	ASIA impairment scale
ASIA	American Spinal Injury Association
BDNF	Bone-derived neurotrophic factor
CNS	Central nervous system
CST	Corticospinal tract
EMG	Electromyography
FES	Functional electrical stimulation
(i)SCI	(incomplete) Spinal cord injury
ISNCSCI	International Standards for Neurological Classification of Spinal Cord Injury
MEP	Motor-evoked potential
PCA	Principal component analysis
(r)TMS	(repetitive) Transcranial magnetic stimulation
SCIM	Spinal cord independence measure
SSEP	Somatosensory-evoked potential
WISCI	Walking index for spinal cord injury

Neuronal Control of Normal and Impaired Locomotion

Locomotion is determined by the electromyographic (EMG) activation of antagonistic leg muscles and intrinsic muscle properties. The EMG activity recorded from the leg muscles reflects the action and interaction between central programs and afferent inputs from various sources, which can be separated to only a limited degree. For an assessment of the neuronal control of locomotion we have to record the EMG activity from several leg muscles and the resulting biomechanical parameters such as joint movements and, eventually, muscle tension. By such an approach it is possible to evaluate the behavior of neuronal and biomechanical parameters and their changes that may lead to a gait disorder. The physical signs obtained during the clinical examination give little information about the pathophysiology underlying a movement disorder: stretch reflex excitability and muscle tone are basically different in the passive (clinical examination) compared to active motor condition (functional movement). In addition, during a movement such as gait, several reflex systems are involved in its execution and control. Therefore, for an adequate

treatment of a movement disorder, we have to know about the function of reflexes and motor centers involved in the respective motor task.

Furthermore, the movement disorder also reflects secondary compensatory processes induced by the primary lesion. In many cases, the altered motor behavior can be considered as an optimal outcome for a given lesion of the motor system (cf. [1]). The complexity of primary and secondary effects of a lesion requires a detailed analysis of movement disorder to define the target of any treatment.

Physiological Basis of Human Locomotion

Leg muscle activation during locomotion is produced by spinal neuronal circuits within the spinal cord, i.e., the central pattern generator (CPG; for a review, see [2]). For the control of human locomotion, afferent information from a variety of sources within the visual, vestibular, and proprioceptive systems is utilized by the CPG. The convergence of spinal reflex pathways and descending pathways on common spinal interneurons seems to play an integrative role (for review see [3]), similar to that in the cat [4]. The generation of an appropriate locomotor pattern depends on a combination of central programming and afferent inputs. The actual locomotor conditions (e.g., slippery surface) determine the mode of organization of muscle synergies [5], which are designed to meet multiple conditions of stance and gait [6]; for a review, see [7].

There are indications for a quadrupedal coordination of human locomotion. During locomotion corticospinal excitation of upper limb motoneurons is mediated indirectly via propriospinal neurons in the spinal cord [8]. This allows a task-dependent neuronal linkage of cervical and thoracolumbar propriospinal circuits controlling leg and arm movements during locomotor activities. Furthermore, a precision locomotor task, such as obstacle stepping, involves a quadrupedal distribution of spinal anticipatory activity for limb coordination [9].

An actual weighting of proprioceptive, vestibular, and visual inputs to the equilibrium control is context dependent and can profoundly modify the central program. Through this weighting, inappropriate movements are largely eliminated (for a review, see [7]). Any evaluation of reflex function has to be assessed in connection with the actual motor program and the biomechanical events, including their needs and their restraints.

Gait Disorder Following Spinal Cord Injury

An incomplete spinal cord injury (iSCI) is followed by a spastic gait disorder. Clinically, spasticity produces numerous physical signs such as exaggerated reflexes, clonus, and muscle hypertonia. Spastic hypertonia has been defined as a

resistance of passive muscle to stretch in a velocity-dependent manner following activation of tonic stretch reflexes [10]. On the basis of clinical observations a widely accepted conclusion was drawn regarding the pathophysiology and treatment of spasticity that exaggerated reflexes are responsible for the observed muscle hypertonia and, therefore, the movement disorder. The function of these reflexes during natural movements and the relationship between exaggerated reflexes and movement disorder are frequently not considered.

The physical signs of spasticity bear little relationship to the patient's disability, which is due to a movement disorder. In patients with a subcortical vascular encephalopathy [11], a spinal cord lesion or a brain lesion [12], a characteristic gait impairment is seen. This can also be in part the consequence of secondary changes in compensation to the CNS lesion. More information about the pathophysiology of spastic gait disorder can be obtained by electrophysiological recordings. There is some difference between spasticity of cerebral origin and that of spinal origin, but the main features, such as leg muscle activation during locomotion and spastic muscle tone, are quite similar [6, 12, 13]. Recording of the electrophysiological and biomechanical measures [14] or a three-dimensional analysis can uncover specific features of the disorder [15]. This may be used as an objective tool to quantify the impairment and treatment effects on gait parameters. An overview of the mechanisms thought to be involved in spastic movement disorder is shown in Fig. 23.1.

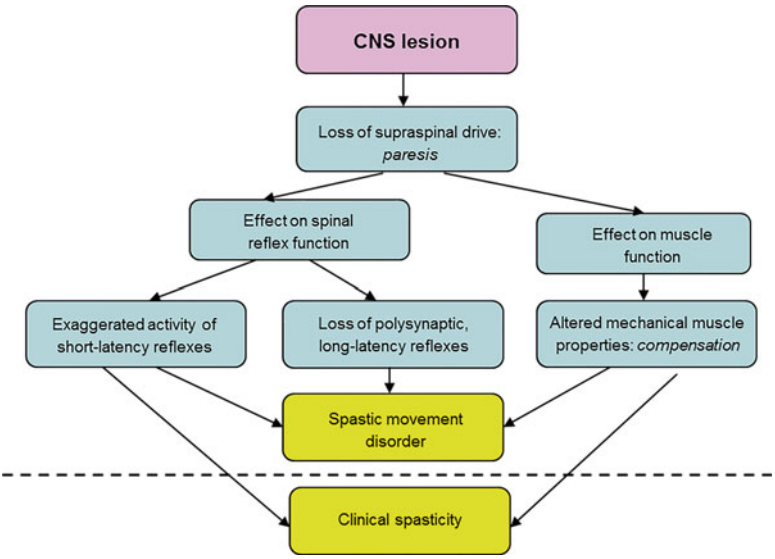

Fig. 23.1 Suggested mechanisms leading to spastic movement disorder and clinical spasticity are depicted. *CNS* central nervous system

Reflexes and Muscle Tone

It has been suggested that neuronal reorganization occurs following central lesions in both cat [16] and human [17]. Novel connections (e.g., sprouting, functional strengthening of existing connections, and removed depression of previously inactive connections) may cause changes in the strength of inhibition among neuronal circuits. In addition, supersensitivity caused by denervation may occur [16]. Recent observations indicate that spinal cord lesions do not cause sprouting of primary afferents in either cat [18] or human [19]. However, a change in the reduction of presynaptic inhibition of group Ia fibers [20], which is stronger with paraplegic compared to hemiplegic patients [21], or a change in transmission in group II pathways [22] might lead to exaggerated tendon tap reflexes. However, no correlation exists between decreased presynaptic inhibition of Ia terminals and the degree of spasticity [21].

The treatment of spasticity is usually directed toward reducing stretch reflex activity as exaggerated reflexes are thought to be responsible for increased muscle tone and, therefore, the movement disorder. Studies on muscle tone and reflex activity have usually been performed under passive motor conditions [23–25]. In patients with spastic paresis muscle hypertonia was found to be more closely associated with muscle fiber contracture than with reflex hyperexcitability [26].

Investigations on functional movements of leg [27–29] and arm [23, 30, 31] muscles have not revealed any causal relationship between exaggerated reflexes and spastic movement disorder (for review see [32]). In patients with cerebral or spinal lesions, the reciprocal mode of leg muscle activation during gait is preserved in spasticity. Exaggerated short-latency stretch reflexes in spasticity are associated with an absence or reduction of functionally essential polysynaptic (or long-latency) reflexes. In addition, both cutaneous [33] and stretch [34, 35] reflex modulation are impaired during walking in patients with spinal cord lesion. It can be assumed that impaired modulation of stretch reflex activity along with increased stiffness of leg extensor muscles contributes to the impaired walking ability in these patients [32].

Corresponding to the degree of muscle paresis during both gait [27] and elbow movements [30], EMG amplitude is smaller compared to that in healthy subjects, most probably due to the impaired function of polysynaptic reflexes. Fast regulation of motoneuron discharge, which characterizes normal muscle, is absent in spasticity [36, 37]. This corresponds to a loss of EMG modulation during gait.

In spastic paresis a fundamentally different development of tension of the triceps surae takes place during the stance phase of step cycle [27]. In the unaffected leg, the tension development correlates with the modulation of EMG activity (as in healthy subjects), while in the spastic leg tension development is connected to the stretching period of the tonically activated (with small EMG amplitude) muscle. During gait there is no visible influence of short-latency reflex potentials on the tension developed by the triceps surae. A similar discrepancy between the resistance to stretch and the level of EMG activity has been described for upper limb muscles of patients suffering spastic paresis [30, 38, 39]. Spastic muscle tone during functional

movements cannot be explained by an increased activity of motoneurons, but instead by a transformation of motor units such that a higher triceps surae tension to EMG activity relationship occurs during the stretching period in the stance phase of gait [32]. Consequently, regulation of muscle tension takes place at a lower level of neuronal organization.

Biomechanical Muscle Transformations

There are several findings that support the suggestion that changes in mechanical muscle fiber properties occur in spasticity. Torque motor experiments applied to lower limb muscles indicate a major, nonreflex contribution to the spastic muscle tone in the leg extensors [35, 40]. Histochemistry and morphometry studies of spastic muscle have revealed neurogenic changes of the muscle fibers [36, 41]. Changes in mechanical muscle fiber properties might also be due to a shortening of muscle length as a result of a decrease in the number of sarcomeres in series along the myofibrils, accompanied by an increase in resistance to stretch [42]. Such muscle contracture can be produced in experimental animals by plaster cast immobilization of muscles in shortened positions. The alteration to a simpler regulation of muscle tension following paresis due to spinal or supraspinal lesions (cf. Fig. 23.1) is basically advantageous for the patient as it enables the patient to support the body during gait and, consequently, to achieve mobility [29].

Therapeutic Approaches

In mobile patients primarily physiotherapeutic approaches should be applied, while antispastic therapy represents a second tool. By a functional, i.e., locomotor training, spastic muscle tone becomes adjusted to a level which is required to compensate for the loss of supraspinal drive (cf. Fig. 23.1). Consequently, the recovery of an appropriate leg muscle activation during the course of a locomotor training is associated with a corresponding reduction in spastic muscle tone [43]. Only in immobilized patients antispastic drugs or intrathecal baclofen infusions may be of benefit to relieve muscle spasms and improve nursing care [13]. Botulinum toxin injections in spastic paretic muscles are associated with both a decrease of stride-time in the paretic leg and a larger range of motion of knee and ankle joints during locomotion [44].

Recovery of Locomotor Function in Human SCI

An incomplete spinal cord injury (iSCI) provides the anatomical requirements for various amounts of functional recovery due to spared fibers bridging the injury site, providing the basis for plastic changes taking place at multiple levels [45, 46].

Locomotor training guides recovery processes and thus enhances functional recovery after central nervous system (CNS) lesions [47–51]. It was shown that training should be task oriented, as improvements occur mainly in the trained activity while untrained functions may remain unchanged [52]. Some adaptations of the motor system occur without affecting behavioral outcomes (e.g., walking), while other changes may be relevant for functional improvement (i.e., gain of visible movement and regain/improvement of ambulatory capacity).

Neurological and Functional Recovery

Examinations according to the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) patients [53, 54] developed by the American Spinal Injury Association (ASIA) reveal that patients' neurological status may change spontaneously within the first year after injury [55–57] and even beyond [58]. According to these reports, sensory-motor complete patients (ASIA Impairment Scale (AIS) A) were reported to have a conversion rate to a neurologically incomplete lesion (AIS B–D) 1 year postinjury of 16.0–32.9 %, depending on the study (Fig. 23.2).

AIS B patients evolve to motor incomplete (AIS C–E) in 60.0–73.0 % of cases. The more affected motor incomplete patients (AIS C) show a rather high rate of conversion to an improved AIS level (D or E, 70.7–84.8 %), while the less affected motor incomplete patients (AIS D) only convert to a higher AIS level in 8.5–15.6 %

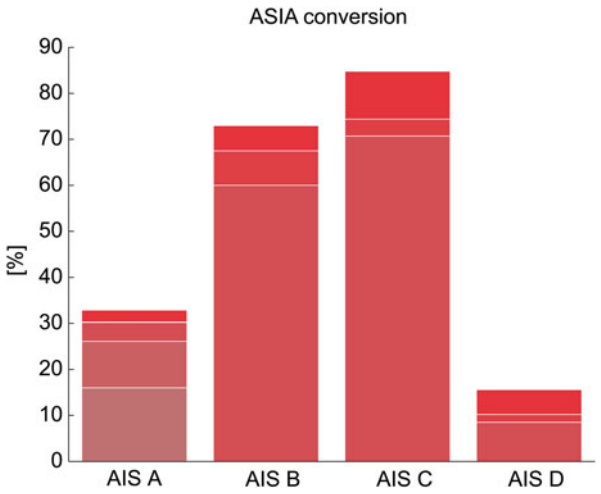

Fig. 23.2 ASIA (American Spinal Injury Association) conversion rates to AIS (ASIA impairment scale) levels greater than initial grade as reported by different studies (represented by different shades of *red*)

of cases. These spontaneous changes of neurological function contain information in several aspects. Firstly, and very immediately, the mentioned results suggest that neurological recovery can occur in the absence of specific, targeted treatment (in addition to conventional therapy) and needs to be considered when investigating the effects of novel interventions in an acute/subacute and even chronic phase. Further, the examinations that compose the ISNCSCI are insufficiently reporting on the number of neuronal connections that were spared from the lesion (i.e., it is highly unlikely that a sensory-motor complete patient converts to an AIS C patient without any residual fibers surviving the injury), and on the other hand, the different rates of AIS conversions with respect to the initial classification indicate that the number of spared tissue and thus the incompleteness of lesion is a crucial prerequisite for a favorable development of rehabilitation, despite the relatively low conversion of AIS D patients to AIS E (= normal). The latter observation probably reflects the incapacity of severed axons to regenerate and the insufficient substitution of lost function by plastic adaptations of uninjured tissue, preventing a complete recovery of iSCI patients back to normal. Interestingly, it has been reported that significant improvements in gait speed after locomotor training occur in 70 % of chronic incomplete SCI patients, while only 8 % showed AIS category conversion [59], suggesting that the AIS is a rather crude tool to classify patients and is insensitive to changes induced by locomotor training [57]. Therefore, if subtle changes in the functional state are to be recognized, different outcome parameters should be chosen. For patients who do not regain any walking function (i.e., AIS A and B patients), measures of ambulatory performance are rather useless. The Spinal Cord Independence Measure (SCIM) is a reliable and valid tool to score the ability of patients to independently perform activities of daily living and also identifies progress in motor complete patients [60–62], not revealing information on underlying mechanisms of functional gain. Likewise, improvements in walking performance are probably achieved by so-called compensatory mechanisms, i.e., mechanisms not attributable to regeneration of severed tissue but plastic changes in uninjured tissue requiring modification of existing motor behaviors and learning of new movement strategies. Recovery of function mediated via compensatory processes was further supported by electrophysiological recordings. AIS conversion is not paralleled by changes in motor- and sensory-evoked potentials (MEPs and SSEPs, respectively), while latencies are particularly robust over time and do not indicate any regeneration or remyelination of fast conducting axons [63].

Therapeutic Approaches

It is quite challenging to discern recovery induced by activity (as part of a therapy regimen or otherwise) from spontaneous recovery alone. Furthermore, it is unethical to conduct a study including a control group of patients who do not receive any kind of therapy. Spontaneous recovery is, therefore, often the result of so-called conventional therapy that takes place during the inpatient as well as the outpatient

phase. However, there is no consensus on the content of *conventional* therapy. In neurological disorders affecting motor behavior a physical therapy typically consists of stand training, balance training, strength training, and locomotor training, while the latter may be performed using a driven gait orthosis (e.g., Lokomat), treadmill training, and overground training with/without assistive devices. A focused training emphasizing on a specific form of locomotor training (i.e., intensive training using driven gait orthoses [64, 65], high-dose treadmill training [66–69], or enforced overground training [64, 70, 71]) exceeding normal amounts of gait-rehabilitation therapy was studied for its effectiveness in improving walking performance. In general, all of the locomotor training approaches lead to some improvements in walking capacity, often not favoring one method over another [64, 72–75].

Promising novel interventions with the aim of improving functional outcome are expected to have effects beyond those induced by any kind of locomotor training. Therapeutic approaches that were examined with regard to their beneficial modulation of motor output included various types of stimulation. Functional electrical stimulation (FES) during walking consisted of lower-limb muscle stimulation at specific time points of a gait cycle (e.g., stance to swing transition, swing to stance transition) and was most often used in combination with locomotor training [76, 77] to induce a withdrawal reflex of the leg and enhance hip flexion during swing. It was shown that the therapeutic effects are higher if the stimulation is applied when an individual attempts to volitionally perform a movement, apparently inducing plastic changes at a cortical excitability or spinal synaptic level [78, 79]. This type of stimulation was also applied in tetraplegic patients in the upper limbs and showed improved grasping performance in comparison to patients who received conventional occupational therapy only [80]. Transcranial magnetic stimulation (TMS) is a noninvasive method used for inducing action potentials in the motor cortex that propagate along the corticospinal tract (CST) to the effector organs. TMS can be applied as single pulses usually used as a diagnostic tool to assess the functional integrity of the corticospinal pathway [63, 81, 82] and to investigate mechanisms of neural control of walking [83, 84]. Repetitive TMS (rTMS) was applied therapeutically in SCI patients for pain relief, reduction of spasticity, and improvement of motor function and is believed to induce plastic changes at cortical, subcortical, and spinal levels [85]. rTMS was applied in iSCI patients to reinforce existing, uninjured pathways and modulate the excitability of the motor cortex [86, 87]. The treatment was shown to improve lower extremity motor strength and walking function and led to a decrease in spasticity in SCI populations [86, 88].

The rather novel approach of electrical epidural spinal cord stimulation for the facilitation of walking revealed very promising results in severely injured SCI rats [50, 89, 90] and even paraplegic patients [91, 92]. Stimulation intensities were chosen at a submotor threshold level in order to not elicit direct motor responses but to excite and engage spinal neurons that were deprived of tonic supraspinal input caused by the injury. The epidural spinal cord stimulation allowed for intensive overground training in rats which led to the regain of voluntary control of hindlimbs, supposedly mediated by sprouting of axons across the midline into denervated

spinal regions [50]. In human SCI subjects, the effects were less dramatic, but remarkable nonetheless. In four motor complete SCI subjects (2 AIS A, 2 AIS B) who received an epidural stimulator, the electrical excitation of spinal networks led to a regain of voluntary movements of lower limbs in a supine position that could not be achieved by preceding several months of intensive motor training [91].

A rather different approach, whose mechanism is not completely understood but which showed interesting results in a chronic iSCI patients' cohort, is the application of intermittent hypoxia combined with overground walking training [93]. This training paradigm led to an increase in walking performance (speed and distance) compared with a control group that just received intermittent hypoxia and a sham group receiving normoxic air. It was shown in preclinical studies performed in rats that intermittent hypoxia induces neuronal plasticity mediated by bone-derived neurotrophic factors (BDNF) and TrkB. This method has the advantage of not being invasive, but frequent hypoxia may bear adverse effects such as high blood pressure [94, 95].

Contributors to Recovery

Functional recovery after SCI is assessed by clinically meaningful scores of walking speed and endurance (10MWT, 6minWT) as well as functional scores (i.e., SCIM, WISCI), which were developed for this specific group of patients [60, 62, 96]. It is, however, not possible to derive conclusive information on underlying processes of recovery from these endpoints. Even in studies assessing more elaborate measures of walking (i.e., kinematic and kinetic gait data), the improvements of particular outcome measures are not unequivocally attributable to identified underlying mechanisms [97, 98]. A comprehensive assessment including a multitude of parameters of various aspects (i.e., function, performance, movement quality, nerve conductivity) imposes challenges with regard to data analysis and interpretation. Most often, the assessor defines meaningful outcome parameters based on the existing literature and own experience in a rather subjective manner. In order to circumvent the bias of preselecting target variables that should best represent recovery, a more objective approach is of need. An unbiased multivariate analysis including measures of different modalities may improve these shortcomings. Principal component analysis (PCA) considers a great number of variables, and parameters that explain the largest amount of variance within the entire set of data can be extracted [99]. With this method the structure and behavior of data identify the measures that are most influential and significant for explaining differences between subclasses, which are built based on data behavior rather than subjectively defined criteria. Using this method, clusters of parameters could be identified that are distinctly controlled in iSCI, probably reflecting distinguishable domains of neural control of walking [100]. Gait-cycle parameters (e.g., step length, cadence, single-limb support phase, etc.) showed similar behavior in iSCI patients and were adequately

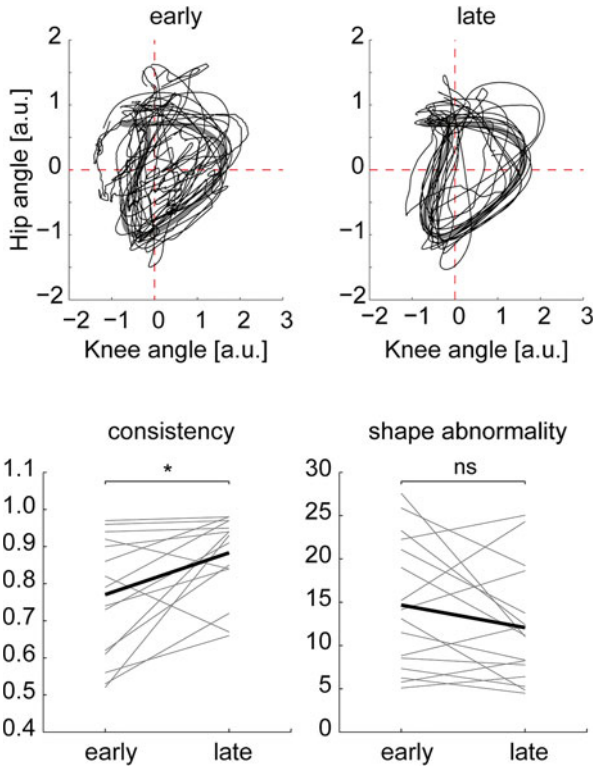

Fig. 23.3 Multisegment intralimb coordination is represented by the spatiotemporally simultaneous coordination of hip and knee angles during a gait cycle. This measure reveals that the gait pattern can be more reliably produced (greater cycle-to-cycle consistency) at a late stage of recovery compared to early walking. In contrast, the shape of the intralimb pattern, and therefore the gait quality, does not approach normal from early to late recovery. *a.u.* arbitrary unit, *asterisk* statistically significant ($p<0.05$), *ns* statistically not significant ($p>0.05$)

speed modulated, which was in contrast to stroke or Parkinson's patients. Measures reflecting more complex lower-limb movements (intralimb coordination) were distinctly altered in iSCI patients and could not be normalized when changing from an unusually slow speed to preferred walking speed, in contrast to healthy control subjects. Interestingly, the intralimb coordination is also relatively indifferent to recovery, meaning that the quality of the pattern is equally unresponsive to improvements in speed over time during rehabilitation, while the consistency of the pattern increases significantly (Fig. 23.3).

Functional recovery is typically quantified by walking speed and distance, which are important determinants for the ambulatory capacity of a person [100, 101]. By what means a patient actually achieves a faster walking speed may subsequently be investigated using linear regression models that identify variables which are responsive to increases in speed. However, many of the gait parameters (e.g., step length

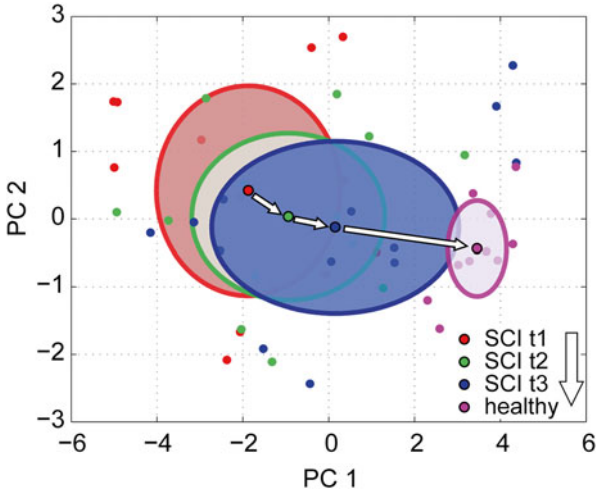

Fig. 23.4 Multivariate analysis of a multitude of gait parameters reveals a convergence of the gait pattern over time (*t*1–*t*3) toward unimpaired in subjects with an incomplete spinal cord injury (SCI). *PC* principal component

and cadence) are sensitive to increases in speed without actually reflecting true recovery (i.e., improvements induced by physiological, anatomical, and/or biological changes). Contributors to recovery may be identified using a multivariate approach (PCA) where no dependent variable needs to be predefined. The analysis yields clusters of parameters that may be mutually interrelated and intricately contribute to a maximally large amount of variance within the data. Surrogate variables (principal components) are composites of the original variables, while the first principal component explains the largest variance. iSCI gait data are clearly distinguishable from healthy data, while a convergence toward healthy takes place over time (Fig. 23.4).

This procedure also revealed that factors contributing to recovery may or may not actually show any improvements. The intralimb coordination, for example, may remain pathological in iSCI patients (i.e., no improvement) while it is a crucial determinant of whether or not a patient shows gait recovery. Three main patterns of recovery could be discerned where gait quality (intralimb coordination) was not necessarily paralleled by the progress in speed. The relative indifference of the intralimb coordination to recovery over time seems to go in line with the evolution of MEP latencies. It appears that the intralimb coordination relies on a relatively large amount of intact supraspinal input (mildly affected patients show a normal pattern while more strongly affected patients show marked alterations [102]). Corticospinal tract integrity as assessed by MEPs may be used as a proxy for the amount of remaining supraspinal input. It may thus be that the complex lower-limb coordination of moderately to severely impaired patients only normalizes if restorative processes take place (i.e., regeneration of severed fibers).

Conclusion

Knowledge of the mechanisms underlying gait disorders and recovery is crucial for targeted interventions with the aim of an efficient improvement of the functional state. The primary lesion may lead to secondary alterations in peripheral tissue remote from the injury and thus additionally determines the characteristics of a gait disorder. Following SCI, spasticity is one factor that strongly influences the walking ability and gait pattern of affected subjects. Physical activity in motor-incomplete SCI subjects was shown to alleviate spastic symptoms while motor complete patients may profit from antispastic medication. However, in patients with severe paralysis lacking sufficient voluntary muscle strength, spasticity may facilitate or even enable walking.

Data from animals have shown that the type of training paradigm may strongly influence plastic reorganization of neuronal pathways and therefore determines the outcome and success of a therapy [50, 103]. Treatment effects are, however, not easy to interpret. They may be masked by spontaneous recovery and probably consist of improvements induced by motor learning as well as central and peripheral plastic adaptations that either reestablish unimpaired walking or improve function by adopting new strategies. It is, therefore, crucial to consider the different modalities of walking by assessing anatomical, functional, and behavioral aspects of locomotion. It was shown that timed walking readouts simply reflect increased walking speed and are well-controlled throughout recovery, while gait quality remained altered in iSCI patients. The differential capacity of neurological patients (SCI, stroke, Parkinson) to control and modulate specific groups of gait measures suggests their distinct control and recovery potential. Knowledge of these processes serves to ameliorate rehabilitative strategies and enables meaningful interpretation of outcome.

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Chapter 24
Neural Prostheses for Neurotrauma

Arthur Prochazka

Abstract Neural prostheses (NPs) are electrical stimulators that deliver electrical stimulation to nerves and muscles to improve function in a variety of neurological disorders. Here we consider the basic components and design of NPs and their mechanisms of action. Some key advances in the development of NPs are discussed, along with descriptions of the successes, limitations, and failures that have been encountered. NPs that improve upper limb (UL) function, postural control, walking, respiration, and micturition are now either commercially available or in development. NPs are increasingly being used in conjunction with other interventions such as drug therapy and exercise training. In future they may be used to maximize the outcomes of biological treatments such as nerve regeneration. The control of the urinary bladder, whether to promote voiding or to counteract incontinence, is a major area of NP research and development. Growing interest in government and commercial circles in the development of NPs should result in a significant increase in the availability and affordability of NPs and an increase in the range of neurological disorders in which they can be usefully applied.

Keywords Neural prostheses • Neuroprostheses • Functional electrical stimulation • Spinal cord • Stroke • Hemiplegia • Tetraplegia • Paraplegia • Foot-drop • UL function • Bladder control • Incontinence • Overactive bladder

Introduction

Neural prostheses (NPs) are electrical stimulators that activate nerves to improve motor or sensory function after neurotrauma. The simplest NPs have preprogrammed sequences of exercise stimulation and are typically used to strengthen muscles. This intervention is called therapeutic electrical stimulation (TES). More complex NPs respond to voluntary commands or feedback from artificial sensors

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and may be used to improve motor function in activities of daily life (ADLs). This intervention is called functional electrical stimulation (FES) or functional neuromuscular stimulation (FNS). Over the last few years, the term NP has increasingly been used to denote FES rather than TES devices.

There is a long history of experimentation with electrical devices that activate nerves. Electrostatic machines were used by clinicians in the late 1700s to elicit muscle twitches [1, 2]. Induction coils, invented in the mid-nineteenth century, enabled trains of electrical current pulses to be delivered, which elicited smooth muscle contractions. Since that time, many types of stimulator have been used to apply electrical stimulation not only to the peripheral neuromuscular system, but also to the brain and spinal cord. The pulse amplitude required to activate muscle fibers is more than 10 times greater than that required to activate the nerve that innervates them, so muscle stimulators are really nerve stimulators. Denervated muscles have activation thresholds 10 times those of nerves, so individuals with lesions that have permanently destroyed the axons of motoneurons in peripheral nerves or the motoneuron pools in the gray matter of the spinal cord, do not benefit from FES.

NPs may be external to the body, delivering trains of electrical pulses to nerves through the skin, or they may be implanted, delivering the current to nerves via implanted electrodes. External and implanted NPs are now either commercially available or in development to assist in upper limb (UL) function, postural control, walking, respiration, and micturition.

Delivery of Electrical Stimulation

Pulse trains may be applied through the skin via self-adhesive, conductive gel electrodes, conductive rubber electrodes coated with gel, or metal plate electrodes covered with a moistened cloth pad. The gel or water makes intimate electrical contact with the surface of the skin, distributing the flow of current evenly and thus avoiding hot spots of high current density that would occur if the bare conductors were pressed onto the skin directly. Such hot spots cause skin discomfort, inflammation, and burns. Implanted electrodes are composed of insulated leads with conductive terminals implanted on or adjacent to nerves. The leads and terminals are made of a biologically compatible metal such as stainless steel or platinum. The terminals may be built into a silastic button, a silastic cuff, or on the ends of an insulated cannula, as in the case of brain or epidural spinal cord stimulators.

Properties of Pulse Trains

A single current pulse lasting 0.1–0.3 ms, delivered to a nerve, causes a single action potential in axons in the nerve. The number of axons activated depends on the amplitude of the current pulse. A pulse of amplitude 0.2 mA, delivered via a nerve

cuff, would typically activate all 150 or so alpha motor axons in a muscle nerve. The threshold to activate the very first axon would typically be about 0.1 mA. If delivered via surface electrodes, pulse amplitudes of up to 50 mA may be required to activate all the axons in a deep-lying nerve, as only a small fraction of the current reaches the nerve, the rest flowing through non-neural tissue between the electrodes.

A single pulse delivered to a motor nerve activates axons in it and thereby elicits a single muscle twitch that reaches peak force after about 1/20th of a second. When such pulses are repeated at a rate greater than 20 pulses/s, the twitches fuse and the muscle contracts smoothly (a "tetanic" contraction). The force of the fused contraction increases with pulse rate, leveling off at about 35 pulses/s. Fatigue sets in more rapidly the faster the pulse rate. So there are two ways of grading muscle force: varying the pulse rate or varying the pulse amplitude.

The electronic circuitry in good quality NPs controls either the current or the voltage of pulses using feedback. The advantage of current control is that the same current is delivered even if the impedance of the electrode–tissue interface increases, for example when pad electrodes slowly dry out. This ensures the same amount of nerve stimulation, but in order to force the same current through a higher impedance, the voltage must increase. Hot spots of high current density may develop and these can cause local skin irritation and if prolonged, skin burns. Good quality current-controlled stimulators automatically shut down if the impedance exceeds a preset value and indeed this is one of the safety criteria set by regulatory agencies such as the FDA. Voltage-controlled stimulators deliver a controlled level of pulse voltage, so if the electrode impedance rises, less current flows and the nerve is less stimulated. This avoids skin burns, but reduces the number of activated axons and, therefore, the muscle force elicited. In cheap stimulators, neither voltage nor current are feedback-controlled, leading to unpredictable function and safety concerns.

Types of NPs

Surface NPs That Enhance Gait

The first ever FES device delivered trains of stimulus pulses via surface electrodes to the common peroneal nerve to correct foot-drop in people with hemiparesis [3]. An under-heel switch detected the moment the heel began to lift at the onset of the swing phase of gait. This triggered stimulation of the common peroneal nerve, activating muscles that dorsiflex the foot, ensuring ground clearance. A device based on this method, the FEPO (functional electrical peroneal orthosis), was commercialized in the 1970s by a group in Ljubljana [4]. Since then, portable foot-drop stimulators of various designs have been used by many thousands of people worldwide. Most users have been people with hemiplegia due to stroke, though some people with spinal cord injury (SCI) have used them too.

Three models of single-channel foot-drop stimulator in the form of cuffs worn below the knee are currently available: the Odstock ODFS [5], the Bioness L300 http://bioness.com/products/l300.htm), and the Innovative Neurotronics WalkAide [6]. The Odstock and L300 devices both use the Liberson technique of an under-heel sensor to trigger stimulation. In the L300, the sensor signal is transmitted wirelessly from the sensor to the stimulator cuff. The Walkaide uses the signal from a tilt sensor in the cuff, thereby avoiding the need to wear shoes to accommodate an under-heel sensor. The Walkaide is also provided with a wired under-heel sensor for cases in which the tilt sensor method is unreliable. Standard physical therapy stimulators equipped with under-heel sensors have also been used as foot-drop stimulators (e.g., the Empi 300PV).

Foot-drop stimulators and mechanical ankle-foot orthoses (AFOs) have been compared in several recent clinical trials [5, 7–9]. In one study, 26 community-dwelling stroke participants had their AFOs replaced for 8 weeks with NESS L300 devices [10]. Physical activity and walking speeds measured at 2 and 8 weeks were not significantly different between the devices, but the participants judged the FES device superior to the AFO. It was suggested that in future studies, outcome measures focusing on stability and effort of ambulation might capture this preference. A randomized controlled trial (RCT) was recently conducted in which 74 chronic stroke participants used an L300 for 30 weeks and 88 participants used an AFO [11]. No significant differences in gait speed, activity, or participation, were found. However, user satisfaction was again significantly higher in the FES group than in the AFO group. It was concluded that "the development of a validated measure of user satisfaction is important to adequately capture the factors that lead to long-term compliance and the subjective experience of the individual with drop foot from stroke." Broadly similar results were obtained in an RCT involving 399 chronic stroke participants, 187 of whom used an Innovative Neurotronics Walkaide for 6 months and 212 used an AFO [12]. FES with the Walkaide was found to be "noninferior to the AFO for all primary endpoints."

FES has long been known to have carry-over or therapeutic effects [13–15]. In the author's experience, some but not all, hemiplegic users have markedly less foot-drop for up to an hour after using their stimulator. This is a potential advantage of foot-drop stimulators over AFOs that has not been emphasized in the comparative studies so far [16].

Three countries with public health care systems have provided reimbursement for foot-drop stimulators: Yugoslavia [17], Denmark (Dr. Benny Klemar, personal communication) and the UK [18]. In the USA, a ruling by the Centers of Medicare and Medicaide Services (CMS) in the 1980s denied reimbursement for neuromuscular stimulators when used for neurological disorders, even though reimbursement was approved for neuromuscular stimulators when used to treat back pain. This is curious, as back pain is often the result of a neurological disorder. A reimbursement code was issued by CMS in 2006 for neuromuscular electrical stimulation with the Parastep system (see below) to improve walking in people with SCI who met a specific, restricted list of criteria [19]. The limited coverage of FES devices has been a crucial barrier to the widespread adoption of foot-drop stimulators in the USA.

A six-channel FES stimulator, the Parastep, was introduced commercially in the 1980s. It is used in conjunction with a walker and controlled by hand switches. Up to six muscles are stimulated (gluteus medius, quadriceps, and the foot dorsiflexors in each leg). It is suitable for people with SCI who have enough arm and hand function to control the device and use the walker for partial weight support. The metabolic costs of using the Parastep for ambulation are high [20–22]. A study in France concluded that "In spite of its ease of operation and good cosmetic acceptance, the Parastep approach has very limited applications for mobility in daily life, because of its modest performance associated with high metabolic cost and cardiovascular strain. However, it can be proposed as a resource to keep physical and psychological fitness in patients with SCI" [23]. The Parastep System is available through the W.A.L.K. Foundation (sigmedics.com) and some Veterans Administration and private clinics (G. Maltezos, personal communication).

In 19 people with chronic incomplete SCI (ASIA C), overground and treadmill locomotor speed more than doubled after 3 months of 1.5 h training 3 days/week in which multichannel FES was combined with partial weight support [24]. The overall conclusion on surface FES for gait after SCI reached in the SCIRE metastudy [25] was that "FES-assisted walking can enable walking or enhance walking speed in incomplete SCI or complete (T4–T11) SCI. Regular use of FES in gait training or activities of daily living can lead to improvement in walking even when the stimulator is not in use."

Surface NPs That Enhance Upper Limb Function

The first detailed studies of electrical stimulation of the UL were also performed by the Ljubljana group [26, 27]. Subsequently, a group at Rancho Los Amigos Rehabilitation Hospital in Los Angeles used therapeutic electrical stimulation to improve hand function in people with subacute and chronic stroke [28, 29]. It was not until the 1990s that portable surface FES devices for hand function were developed. These were in the form of an instrumented splint: the Handmaster [30, 31], a fingerless neoprene garment, the Bionic Glove [32, 33], and the ETHZ Paracare [34, 35].

Since 2005 a modified version of the Handmaster has been commercially available in the USA under the name Bioness H200. Originally, it comprised a hinged wrist-splint with a separate stimulator electrically connected to the splint via a cable. Electrodes attached to panels inside the splint delivered trains of stimuli to three or four motor points of muscles in the forearm and hand. In 2014, a new version was introduced, the Wireless H200, in which the stimulator is built into the splint and controlled by a separate wireless remote controller. A study of the original Handmaster in eight individuals with C5–6 SCI reported significant improvements in hand function after 3 weeks of daily usage [36]. Its size and structure make it suitable mainly for use as a therapeutic aid to exercise therapy, rather than as an orthotic device intended for use in ADLs.

The Bionic Glove was a fingerless neoprene gauntlet with an in-built stimulator [32]. Metal mesh panels inside the garment made contact with self-adhesive gel

electrodes previously placed over motor points of the forearm and thenar muscles. The device incorporated a wrist angle sensor. Voluntary wrist flexion triggered hand opening stimulation and wrist extension triggered grasp, a control strategy designed to augment tenodesis grasp and release. In a pilot study in 9 people with C6–7 SCI, grasp force increased fourfold and performance of ADLs improved significantly during stimulation [33]. In another study in 12 people with C5–7 SCI, after 6 months of using the Bionic Glove in ADLs, voluntary hand function in the absence of the device had improved [37]. Individuals with C6–7 SCI benefited the most. High-functioning participants tended to use the device less than those with low to medium levels of function, because they did not gain enough additional hand function to offset the time it took to don and doff the device.

The Bionic Glove was superseded by elastic gauntlets or wristlets that incorporated wettable pad electrodes clipped to conductive studs on the inside surface of the garment. Users switched stimulation voluntarily from hand opening to grasp with the use of a wireless earpiece that detected either toothclicks [38] or head-nods. The toothclick version was used in recent telerehabilitation studies of FES-assisted UL exercise in people with chronic tetraplegia [39] or chronic hemiplegia [40]. A head-nod-controlled wristlet with an optional thenar extension for tetraplegic users is in the final stages of commercialization by Rehabtronics Inc. and should be available by the end of 2015 (Fig. 24.1). Because of its relative simplicity, it will

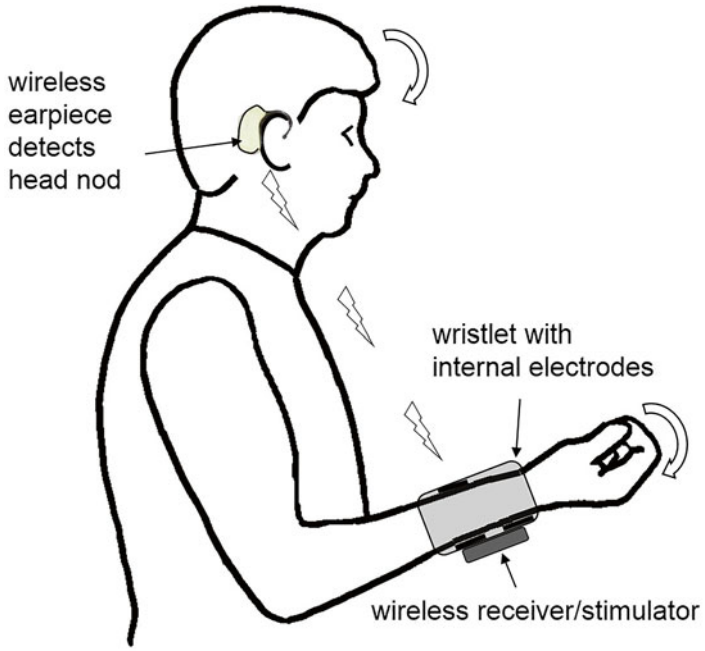

Fig. 24.1 Hand stimulator controlled by a wireless earpiece. A sensor in the earpiece detects voluntary head nods. A radio-frequency signal is transmitted to a receiver in a stimulator attached to a neoprene wristlet. The signal triggers a train of stimuli applied to forearm muscles via wettable pad electrodes inside the wristlet. Sequential head nods elicit hand opening, closing, and release

be more affordable than the H200. It can be worn under a shirtsleeve. The discrete form factor and the intuitive, hands-free method of triggering should make it useful in ADLs, in addition to its role in exercise therapy.

Multichannel upper limb FES with surface electrodes has been tested in people with C3–7 tetraplegia [41]. The stimulator was programmed to activate proximal and distal muscles in a sequence to enable reach and grasp. One of the problems with surface stimulation of the large proximal muscles of the arm is that during joint rotation, their motor points can move several centimeters under the skin. Consequently, the level of muscle activation elicited from an electrode attached to the skin tends to vary with the position of the arm, which can make the task of controlling movements accurately and stably very challenging. Nonetheless, encouraging therapeutic results were reported and the device is now commercially available at http://www.myndtec.com/myndmove.

Therapeutic Carry-Over Effects

TES and FES in people living with stroke and SCI have been shown to have carry-over therapeutic effects [13–15] especially when performed in association with voluntary exercise training [39, 41–47]. Carry-over effects lasting a few hours may result from short-term changes in the energetics of neuromuscular activation, whereas carry-over effects lasting weeks or months have been attributed to muscle strengthening, neural plasticity, or both [48–50].

Implanted NPs

The first implantable electrical stimulators were cardiac pacemakers [51]. These stimulate specialized muscle fibers in the heart and are therefore not strictly NPs. However, the technology that had to be developed in order to safely and reliably stimulate heart tissue provided a basis for the development of numerous NP devices in the subsequent decades. The most successful implantable NP is the cochlear stimulator (Clark et al. 1977). Over 50,000 multichannel cochlear stimulators were implanted in the 1990s (Clark 1999; Kessler 1999). Other implantable NPs include dorsal column stimulators for pain control and spasticity [52], deep-brain stimulators to treat movement disorders [53–55], and bladder stimulators (Bradley et al., 1963; Stenberg et al. 1967). In 1997, the Medtronic Interstim sacral nerve root stimulator was approved by the FDA to treat urge incontinence. According to Medtronic, 40,000 Interstim devices have been implanted worldwide. The Interstim has also been implanted off-label in SCI individuals to facilitate voiding (see below).

Implanted NPs That Enhance Gait

The development of implantable NPs to restore limb movement has been relatively slow. The task is difficult: trains of pulses of just the right duration and intensity must be delivered to nerves innervating one or more muscle groups, in such a way as to coordinate the segments of the limb to safely produce functional movements in the face of variable loading and terrain. The simplest application is foot-drop, because here the main task is simply to dorsiflex the foot to clear the ground during the swing phase of gait. A small number of hemiplegic people were implanted with foot-drop stimulators in pilot studies in the 1970s and 1980s [56–58]. Medtronic then developed the "Neuromuscular Assist" system in which an under-heel sensor was used to wirelessly trigger an external control unit that delivered power and stimulus commands to an implanted receiver via a coil antenna taped to the skin. The receiver delivered pulse trains to the common peroneal nerve through a pair of electrodes in a silicone rubber flap wrapped around the nerve distal to the knee [59]. The system worked well in 25 of the 31 recipients for at least 7 years but Medtronic decided not to pursue commercialization.

Recently, two types of implantable foot-drop stimulators have become available commercially in Europe, the Finetech STIMuSTEP (www.finetech-medical.co.uk) [60, 61] and the Neurodan ActiGait (www.neurodan.com). Ten people with hemiplegic foot-drop were reported to have been implanted with the STIMuSTEP and 15 with the ActiGait system [62], nearly all of whom showed significant improvements in gait [63]. Technical problems occurred, but were resolved at follow-ups [64]. Like the Medtronic Neuromuscular Assist device, the STIMuSTEP and ActiGait stimulators are triggered from an under-heel sensor. A fully implanted system that uses sensory signals from the foot to trigger stimulation of the common peroneal nerve has been tested clinically but as yet has not been commercialized [65–67].

Gait deficits after SCI are generally too complex to be treated with foot-drop stimulators alone. NPs that stimulate up to 16 leg muscles have been developed and tested [68–71]. The results of the first clinical trials have been encouraging, particularly in relation to posture, standing [72], and the avoidance of pressure ulcers [73–77].

Epidural and Intraspinal Stimulators

Epidural stimulation of the lumbar spinal cord with electrodes placed on the dorsal aspect of the dura mater of the spinal cord has been explored in clinical trials as a means of boosting residual locomotor function after incomplete SCI [78–83]. There is evidence that epidural stimulation facilitates locomotion by activating sensory axons in dorsal roots and the dorsal columns [84, 85]. It has been posited that the sensory input increases the general level of excitability of spinal locomotor circuits [46].

Another experimental approach explored in recent years is intraspinal microstimulation (ISMS). Pulse trains delivered through microwires implanted in the lumbosacral enlargement of the cat spinal cord were shown to activate single muscles or groups of synergistic muscles [86–89]. Technical difficulties have been encountered in ISMS implants in spinalized animals, including problems of electrode placement, migration, and tissue damage [90]. After some weeks the movements elicited by ISMS tend to change from the desired synergies to co-contraction of antagonists [91]. Even if this occurs, residual voluntary movement may be boosted by low-intensity ISMS eliciting a generalized increase in excitation of spinal neuronal networks [92, 93], as suggested above for epidural stimulation. In a recent study in monkeys in which a hand was paralyzed by a temporary blockade of the motor cortex, premotor neuronal activity was used to enable some voluntary control of the hand through ISMS [94]. So far, however, no human trials of ISMS have been performed for the restoration of limb movements.

Implanted NPs That Enhance Upper Limb Function

An implanted multichannel stimulator to restore UL movements after SCI was developed in the 1980s and 1990s at Case Western Reserve University [95]. It consisted of a cardiac pacemaker-like implant with leads terminating in electrodes that were sewn to the epimysium of muscles in the forearm and hand. Signals from shoulder or wrist movement sensors were used to provide the recipients with voluntary control over muscle stimulation. The implant received energy and commands by inductive coupling from an external control box connected to a coil taped to the skin. The external controller generated synergies to produce different types of hand movement. The system was approved by the FDA in 1997 and commercialized as the "Freehand System," about 200 of which were implanted in people with C4–C5 tetraplegia. Though the technology was highly advanced and many recipients benefited significantly [96], the device was discontinued in 2002.

There is an interesting analysis of the development of this and other NPs that were clinically effective, but did not survive commercially [97]. The analysis concludes "While the over-exuberant health care spending of the 1970s and early 1980s has taught policymakers a valuable lesson, the vicious cost-containment initiatives characteristic of current Medicare policy are outdated and have far-reaching negative effects on public health. The Centers for Medicare and Medicaid Services should replace their anti-technology bias with a payment system capable of recognizing the profound health economic benefits of neuroprostheses."

It is a challenge to commercialize complex implantable NP systems such as the Freehand, because they have a relatively small market in relation to the development cost [98]. An interesting initiative to emerge from the Neurocontrol/Freehand experience is the formation at CWRU of a nonprofit institute to support the transfer into clinical practice and the subsequent long-term maintenance of this type of NP system after implantation: http://casemed.case.edu/ifr.

Simpler implantable NPs for UL function include the Finetech STIMuGRIP [99] and the Stimulus Router [100]. The STIMuGRIP comprises an implanted stimulator with two pairs of platinum epimysial electrodes that are secured over motor points in the forearm. The user wears an external controller unit strapped to the affected limb, directly over the implant site. The implant receives its power inductively from the external controller. A three-axis accelerometer in the controller detects voluntary movement of the forearm. This activates pulse trains from the implant that elicit wrist extension and hand opening [101]. Three hemiplegic people have been implanted. Three tetraplegic people have been implanted with the StimRouter system, one procedure having been described in a detailed case report [102]. Cuffs were implanted on three forearm nerves controlling hand opening and grasp. The leads from the cuffs terminated under the skin proximal to the wrist. The user wore a neoprene wristlet containing a control unit and moistened pad electrodes. Pulse trains were delivered from the controller via the pad electrodes through the skin to the subcutaneous conductive ends of the leads. The leads delivered the pulses to the nerves. Hand opening and grasp were triggered sequentially with voluntary tooth-clicks, detected by a wireless earpiece.

NPs for Bladder Control

Poor bladder control has been voted the second worst problem, after loss of sex function, among people with paraplegia and the fourth worst problem for those with tetraplegia [103]. Incontinence may occur due to paralysis of the external urethral sphincter. Urinary retention may occur because of the development of bladder sphincter co-contraction (dyssynergia). This can result in episodes of very high bladder pressure leading to vesico-ureteral reflux and renal failure. This was the leading cause of death after SCI before the adoption of clean intermittent catheterization [104].

Electrical stimulation to restore bladder control has been delivered experimentally to the inside of the bladder, the bladder wall, thigh, pelvic floor, dorsal penile nerve, pelvic nerve, tibial nerve, sacral roots, sacral nerves, and the spinal cord. The successes and failures of these approaches have been reviewed [105].

Electrical stimulation of sacral anterior roots was shown in experiments in spinalized animals to elicit voiding [106–108]. Human trials of a sacral anterior root stimulator implant (SARSI) then followed [109] and the device was commercialized (http://finetech-medical.co.uk/en-us/aboutus.aspx). The SARSI system has been implanted in over 2500 people, in some cases for over 20 years [110]. Sacral anterior root stimulation activates the external urethral sphincter and the bladder. Voiding is achieved in bursts by taking advantage of the slower relaxation time of the detrusor (bladder) muscle after short trains of stimuli. For this to work properly, however, reflex contractions of the sphincter must be abolished by cutting the sensory nerve roots (dorsal rhizotomy). This results in an irreversible loss of sensory input from the pudenda and legs. People with SCI who hope for a biological “cure”

are often reluctant to take this irrevocable step. Trapezoidal pulse waveforms can activate the bladder more selectively, thus improving the performance of SARSI implants [111].

Regarding ISMS for bladder control, experiments in animals and humans in the 1970s showed that stimulation through pairs of electrodes implanted in the sacral spinal cord could elicit bladder contractions, but not without co-activating the external urethral sphincter [112, 113]. In more recent studies, microwires were implanted in the dorsal commisure of the spinal cord, which contains interneurons that inhibit motoneurons innervating the external urethral sphincter [114]. Stimulation through these microwires elicited reductions in intraurethral pressure in some trials, but more often, *increases* in pressure were elicited [105, 115]. This was attributed to the dorsal commisure containing more interneurons that excite motoneurons innervating the external urethral sphincter than those that inhibit them [116, 117]. In another ISMS study, voiding was produced in two of three spinally transected cats by stimulating mainly within the dorsal columns [118]. These trials were performed during deep Propofol anesthesia, which suppresses urinary tract responses [119]. Bladder and sphincter responses to ISMS and pudendal nerve stimulation elicited under deep anesthesia in spinal cord-transected cats can change dramatically when the same animals are awake [120]. Further evidence in awake SCI animals would be needed before clinical trials in humans could be justified.

Other less invasive approaches to bladder control with NPs have been explored, for example selective stimulation of branches of the pudendal nerve to inhibit dyssynergia and facilitate bladder contraction and voiding [121–125] and high-frequency blockade of the pudendal nerve to inhibit the EUS [126–132]. It has recently been shown that activation and blockade of the pudendal nerve can be achieved with the stimulus router system in the awake spinalized cat (Fig. 24.2). This may provide a low-cost type of NP for either maintaining continence or eliciting voiding, as only one or two leads would be implanted [131, 132].

NPs for Overactive Bladder

Overactive bladder syndrome (OAB) is defined as "urgency, with or without urge incontinence, usually with abnormal frequency (8 or more voids a day) and nocturia" [133, 134]. Urgency is a strong, sudden desire to void that cannot be postponed and that interrupts daily activities. Urge incontinence is involuntary leakage of urine associated with urgency [135]. Nocturia refers to episodes of urinary urgency interrupting sleep. About 17 % of all people are afflicted with OAB. This amounts to about 60 million people in the USA [136, 137] with an economic cost of over $12 billion [138]. Men and women are equally afflicted, though prevalence increases at a younger age in women (44 vs. 64). Women tend to have more severe symptoms and more adaptive (coping) behaviors [137, 139].

The first-line treatment of OAB is "bladder retraining" [140]. The second-line treatment involves antimuscarinic drugs such as solifenacin (Vesicare). Common

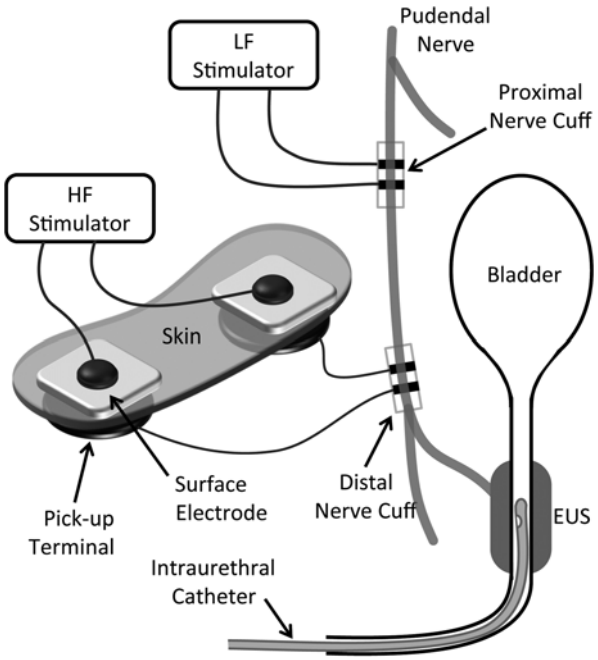

Fig. 24.2 Transcutaneous high-frequency (HF) current delivered to the pudendal nerve at the distal nerve cuff blocked transmission of low-frequency (LF) test pulses elicited at the proximal cuff. An intraurethral catheter was positioned with its side-port in the region of the external urethral sphincter to measure intraurethral pressure elicited by the LF stimulation. Reproduced with permission [162]

side effects are dry mouth, dry eyes, and constipation [141]. These side effects can become intolerable: between 30 and 77 % of people with OAB discontinue their antimuscarinic drugs within a year [142, 143]. The third-line treatment is sacral nerve stimulation (SNS), for example with the Medtronic Interstim implant [144, 145]. The American Urological Association guidelines support SNS in "a carefully selected patient population characterized by severe refractory OAB symptoms or patients who are not candidates for second-line therapy and are willing to undergo a surgical procedure." These devices are expensive and about 25 % of implants result in adverse events requiring hospitalization [146]. Surgical revisions or explanation are fairly frequent [147, 148].

Transcutaneous electrical nerve stimulation (TENS) applied to the posterior tibial nerve (PTN) through surface electrodes above the ankle was shown 30 years ago to reduce the frequency and severity of OAB symptoms [149]. The PTN contains sensory and motor axons of the L4–S3 spinal roots, the same spinal cord segments that control the bladder and urethral sphincters. The method that was subsequently most studied and adopted clinically was percutaneous stimulation, in which an insulated needle is introduced through the skin so that its uninsulated tip lies adjacent to

the nerve [150]. Stimulus pulses are passed between this electrode and a surface electrode, typically applied to the sole of the foot. The system used most widely is the Urgent PC Neuromodulation System (Uroplasty Inc.), approved by the FDA in 2000. Percutaneous PTNS is usually administered weekly for 12 weeks in single 30 min sessions. If OAB symptoms improve, maintenance PTNS once every 3–4 weeks is provided [143, 151]. A recent RCT on 220 participants concluded that the data "provide level 1 evidence that percutaneous PTNS therapy is safe and effective in treating OAB symptoms" [136, 143, 152]

Researchers at the Vrije University in Amsterdam have implanted a Uroplasty Urgent-SQ PTNS stimulator in eight people [153–155]. Five recipients reported improvements in OAB symptoms and quality of life. Three were still using their devices regularly 9 years postimplantation [156]. The development of this implanted device was motivated by the idea that if users were able to deliver PTNS more frequently in their home environment, they would be able to control their OAB symptoms better. Furthermore, since there is some evidence of immediate inhibition of detrusor activity by PTNS [157, 158], users might be able to apply PTNS at the first onset of an OAB event, thus reducing urgency and leaks (though in a small study in multiple sclerosis patients, immediate inhibition of bladder contractions was not observed [159]). The Urgent-SQ implant continues to be pursued commercially, which shows the importance of the goal of providing people with OAB with devices they can use conveniently and on demand in their daily lives.

NPs That Block Nerve Conduction to Reduce Spastic Hypertonus

The high-frequency blockade of overactive pudendal nerves was mentioned above in relation to bladder control. Another interesting possibility explored in recent years is the reduction of spastic hypertonus by reversible high-frequency blockade [160–162]. For example, an NP that caused a transient block of the flexors of the hemiplegic wrist and hand could reduce flexor hypertonus enough to allow unimpeded voluntary hand opening. One side-effect that remains to be overcome before human trials could commence is that at the onset of high-frequency stimulation, many or all of the axons, including nociceptive afferents, fire action potentials and this would probably be painful. Methods have been explored to minimize this side-effect [160, 163, 164].

Another possibility is the partial ablation of nerves with direct current (DC) [165–167]. This could provide a controlled reduction in hypertonus of selected muscles lasting several months [166]. It could be that some months after DC nerve ablation, though efferent axons may regenerate fully [166], afferent axons mediating hyperreflexia may not regenerate nearly as well [168]. These two outcomes could result in a permanent reduction in hypertonus without loss of voluntary force (Cope et al. 1994).

Concluding Remarks

The influential online site www.ebrsr.com (Evidence-Based Review of Stroke Rehabilitation) concludes that "There is strong (Level 1a) evidence that FES treatment improves upper extremity function in chronic stroke." The companion site for SCI www.scireproject.com (Spinal Cord Injury Research Evidence Project) concludes that "The use of neuroprostheses appears to have a positive impact on pinch and grip strength and ADL functions in C5–C6 complete tetraplegia, however, access to the devices (is) limited and (they) continue to be expensive in use." Much the same could be said of the other clinical applications of NPs discussed in this chapter. The use of NPs in hospitals and in the community has been growing steadily over the last decades and the technology continues to improve. The trend to more affordable NPs should further enhance their cost-effectiveness and widespread availability over the coming years.

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Chapter 25
Why Is Functional Electrical Stimulation Therapy Capable of Restoring Motor Function Following Severe Injury to the Central Nervous System?

Mary K. Nagai, Cesar Marquez-Chin, and Milos R. Popovic

Abstract Injury to the central nervous system (CNS) often results in the loss of motor and sensory activity with a tragic impact on quality of life. The anatomic and cellular complexity of the nervous system limits its ability to repair itself, making the effects of the injury permanent. To date, the majority of attempts to restore normal function after damage to the brain or spinal cord have been unsuccessful. Recent studies have demonstrated significant improvements in voluntary motor function in patients with chronic and subacute stroke and spinal cord injury (SCI) using functional electrical stimulation (FES) therapy. In this therapy, patients are asked to perform multitudes of specific motor tasks. During each session, the therapist instructs patients to perform a specific movement at a time, and, after a few seconds of trying, highly controlled electrical stimulation is applied to facilitate that specific movement of the paralyzed limb. After completing this therapy program, individuals are often able to perform the tasks voluntarily, i.e., unassisted by the FES system. Using this approach, we have been able to assist patients with complete and incomplete spinal cord injuries, severe stroke, and pediatric stroke to recover the ability to reach, grasp, stand, and walk. In this chapter, we explain why we believe FES has achieved such extraordinary results.

Keywords Functional electrical stimulation • Functional electrical stimulation therapy • Stroke • Spinal cord injury • Rehabilitation • Neuroplasticity

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Introduction

Disruption of the neural circuitry of the central nervous system (CNS) has potential catastrophic quality of life consequences for an individual who sustained the injury, regardless of the etiology. The complexity of the anatomical organization and the microscopic diversity of neuron types that make up the nervous system pose a major fundamental challenge for self-repair and self-regeneration. The complex organization of the brain increases its sensitivity to even minor intrinsic and extrinsic perturbations. The prognosis for the recovery of functional motor and sensory loss following a CNS injury or neurodegenerative disease is frequently difficult to predict. Despite an explosion of research in restorative neurology in the recent years, we have not yet been able to successfully repair the affected parts of the CNS and restore normal functional motor and sensory activity.

However, one strategy, in particular, is receiving increasing attention because of its ability to repeatedly achieve successful restoration of voluntary upper and lower limb motor functions in severely disabled individuals. More specifically, in the recent years evidence has emerged that functional electrical stimulation (FES) therapy is capable of improving and restoring voluntary motor function in patients with chronic and subacute stroke and spinal cord injury (SCI). In this chapter, we will try to explain why FES therapy has achieved such extraordinary results to date for both stroke and SCI individuals.

Functional Electrical Stimulation

Functional electrical stimulation (FES) technology is able to to produce functional movement in paralyzed muscles after damage to the CNS including spinal cord injury and stroke [1–9]. The artificially created movement is generated by delivering electrical pulses that generate action potentials in muscle and nerve cells producing a muscle contraction [8, 9]. Careful application of highly controlled stimulation sequences makes it possible to produce complex movements such as grasping and walking (Fig. 25.1).

The stimulation can be delivered transcutaneously using electrodes placed on the skin above the nerve of the muscle to be stimulated, making the process convenient and inexpensive. However, transcutaneous stimulation may be incapable of reaching deep structures such as the nerves innervating hip flexors. This limitation can be overcome, to a certain extent, using electrodes in an array configuration, which increase stimulation selectivity by using several contacts [10–12]. It is also possible to use percutaneous and implanted electrodes to apply the stimulation. Percutaneous electrodes are thin wires inserted through the skin and suitable only for short-term FES as they are prone to infection. Implanted electrodes are placed surgically inside the body where they often stay for the rest of the person's life. Subcutaneous (percutaneous and implanted) electrodes may have higher selectivity for stimulation

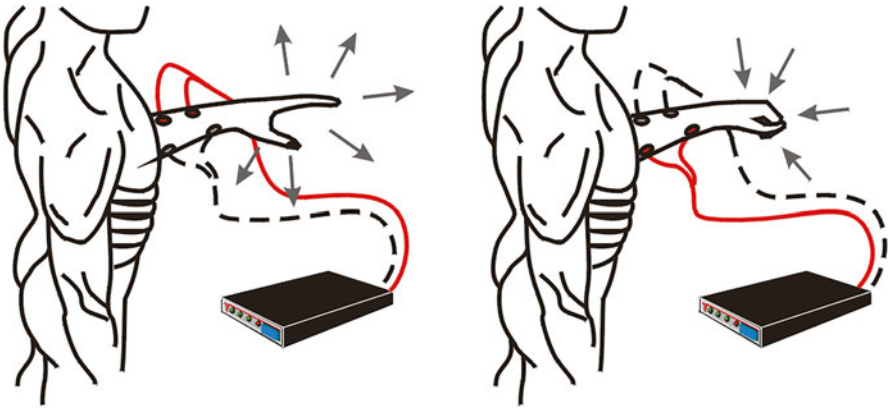

Fig. 25.1 Conceptual depiction of a transcutaneous (noninvasive) functional electrical stimulator used to facilitate hand opening and closing (palmar grasp)

and their electrical discharges can be smaller than those required with surface electrodes. An important disadvantage of implanted electrodes is that they require an invasive procedure to be introduced to the body, and, as with any surgical procedure, there is a risk of infection.

Originally, FES was envisioned to be used as an orthotic device intended to be worn permanently with users activating it whenever required. Important examples of FES-based orthoses include the Parastep [13, 14], which is designed to facilitate walking by applying electrical stimulation to the surface of the lower limbs over the quadriceps and peroneal nerve, and the Freehand system [15], which is permanently implanted in the users' upper limb and produces grasping movements. Both systems were the first devices of their kind to receive FDA approval.

Functional Electrical Stimulation Therapy

As discussed above, most traditional FES programs require the persistent application of the electrical stimulation to provide the individual with functional motor activity. Since 2001, our group has been developing an alternative method for using FES technology. Our FES therapy program requires the individual to attend a finite number of FES therapy sessions. Upon completion of the program the individual will have recovered partial or complete voluntary motor function in their upper or lower extremity [16–27]. Our FES therapy program is designed to "retrain" the injured neuromuscular system through repetitive performance of task-specific exercises. We use FES during these "training" sessions to provide assistance with the components of the task that the individual is unable to perform independently. The assistance provided by the FES system to accomplish each task during the "training" session is determined for each individual task at each therapy session.

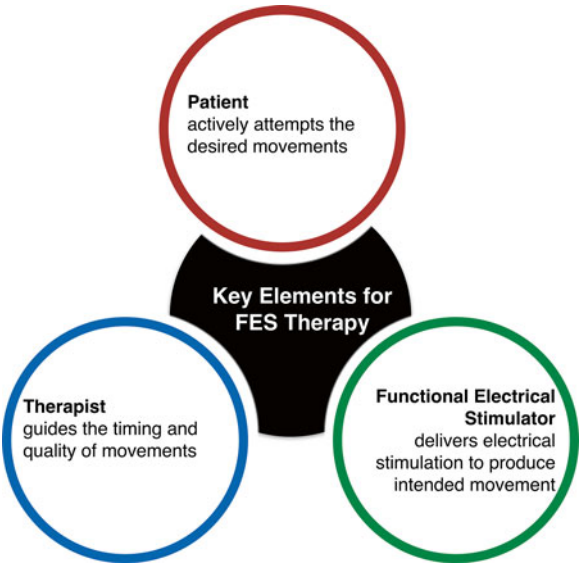

Fig. 25.2 Delivery of functional electrical stimulation therapy for improving reaching and grasping in patients with severe upper limb deficit following stroke

At the completion of the FES therapy program, the individual is usually able to perform the tasks unassisted or with minimal assistance. We have successfully used our FES therapy program to assist adults with incomplete and complete SCI and severe stroke and pediatric stroke patients to recover sustained reaching and grasping motor function [16–18, 20–24]. Adults with an incomplete SCI have also enjoyed robust sustained recovery of functional standing and walking ability after completing our FES therapy program [19, 25–27] (Fig. 25.2).

In our FES therapy program, the participant must attempt to initiate or execute the specific motor task unassisted, such as pinch grasp. Once a brief (10–15 s) attempt to perform the specific task has been made, the therapist delivers an external electrical pulse to the muscles to assist the individual to complete the task. Multitudes of different reaching and grasping tasks are trained. Each task is slightly different and trained for 5 to 7 min. During the early stages of FES therapy, performance of the entire task is supported by FES. As the therapy progresses, FES assistance is slowly reduced and eventually phased out. We believe that the combination of (1) active participation of the patient during therapy, (2) the way in which FES system generates the movement, (3) the fidelity of the movement performed using FES, (4) the accuracy with which FES system mimics the natural limb movements, and (5) repetitive FES-induced movements are critical ingredients of this therapy.

A few groups around the world conduct research on the efficacy of FES therapy, focused primarily on restoration of lower and upper limb function (walking and grasping). In the next few paragraphs, we have described some of the most representative and important work in the field complementary to our work (Fig. 25.3).

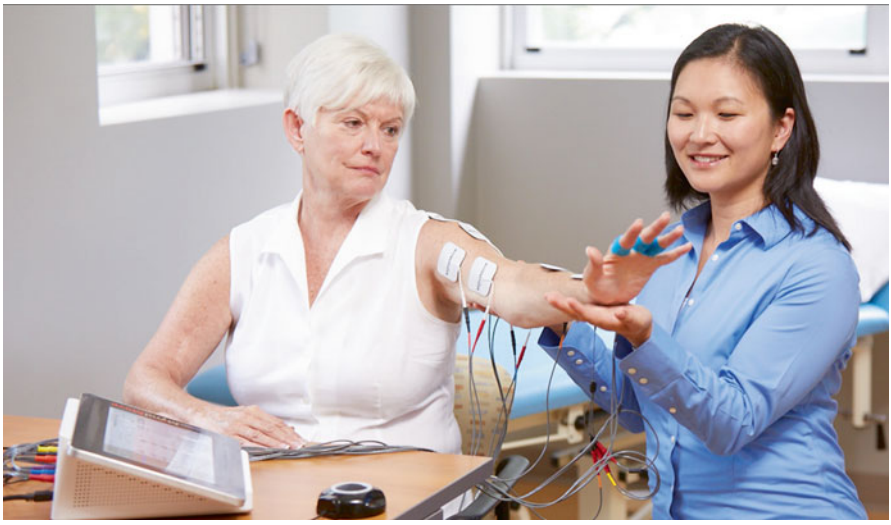

Fig. 25.3 In FES therapy, patients attempt to perform a task, and, after 10–15 s of trying, highly controlled electrical stimulation is applied to facilitate movement of the paralyzed limb. A therapist ensures the quality of the resulting movements

FES Therapy for Lower Limb in Stroke

A common complication experienced among stroke patients is drop foot. Drop foot is the lack of ankle dorsiflexion during the swing phase of gait, resulting in foot slapping and a shortened stride length. It has been shown that a drop foot stimulator that electrically stimulates the common peroneal nerve just before a heel off phase of the gait cycle results in contraction of the muscles responsible for dorsiflexion, effectively compensating for the drop foot during the swing phase of the gait cycle. Drop foot stimulators, including the WalkAide [28] (FDA approved) and Odstock [29] surface stimulators, have been used as orthoses as well as to deliver FES therapy, in which they have repeatedly proven more effective in increasing walking speed by up to 28 % while decreasing physiological cost index (PCI) in hemiplegic stroke patients [28, 29] when compared to conventional therapy. It is important to mention that in addition to these positive results, some studies have not found improvements in walking after FES therapy [30, 31].

FES Therapy for Lower Limb in SCI

In contrast to stroke patients, SCI often results in impaired function not only of the ankle joint but also of both legs, pelvis, and the trunk. Accordingly, the FES systems used to assist walking after SCI target muscles on the whole lower limb. One

of the most commonly used methods for restoring gait in individuals with paraplegia was developed by Kralj et al. [32] in which electrodes are placed bilaterally over the quadriceps muscles and peroneal nerves. Standing is produced by stimulating the quadricep muscles. Users can initiate walking using buttons placed on a walker. The swing phase starts by interrupting the stimulation to the quadriceps and stimulating the peroneal nerve on the same leg. This stimulation is applied rapidly to trigger the flexor withdrawal reflex producing hip and knee flexion and dorsiflexion. Alternate activation of the right and left legs results in gait. In addition to the drop foot stimulators mentioned above, some of the FES systems that use this strategy include Parastep [13, 14] (mentioned earlier), HAS [33, 94], and the RGO [34] which incorporate active and passive braces, and the Case Western Reserve University (CWRU)/VA neuroprosthesis [35–38], which is implanted surgically.

Bajd et al. [39] first reviewed the effect of FES therapy applied to the lower extremities of people with SCI and concluded that it has important therapeutic effects including strength training, and it benefits drop foot and plantar flexor during gait. In addition, Wieler et al. [40] found that the walking speed increased in SCI individuals by 20 % with a drop foot stimulator after FES therapy.

FET for Restoration of Upper Limb Function Following Stroke

Upper limb function is often affected after a stroke. There are many FES systems to help stroke patients compensate for lost grasping [41–52] as well as reaching and grasping functions [18, 53–56]. The effectiveness of FES therapy to improve hand function after stroke has been studied extensively. In 1996, a meta-analysis concluded that FES is effective in promoting recovery of muscle strength [57] and several studies since then, including randomized control trials, have a found positive effect in both the acute [18, 42, 46, 47, 56, 58] and chronic [17, 18, 41, 43, 45, 51, 52, 59] phases of stroke.

Important examples of FES devices used in these studies include the Freehand system [15], the NESS H200 (previously known as NESS Handmaster) [45], the Bionic Glove [46, 49, 59], the ETHZ-ParaCare neuroprosthesis for grasping [55, 60], the devices designed by Rebersek and Vodovnik [50], the Belgrade Grasping-Reaching System [53]—all of them capable of delivering stimulation with surface electrodes—and the Compex Motion neuroprosthesis developed to deliver a variety of reaching and/or grasping protocols [55]. In addition, Chae et al. [41–43] have used a percutaneous system to conduct their work. The NESS system [45, 61] and the new version of the Bionic Glove [46, 59, 62] have been tested recently for self-administering FES therapy at home instead of the usual delivery of the treatment by a therapist in a clinical environment.

FES Therapy for Restoration of Upper Limb Function following SCI

There is little existing research on the use of FES therapy for upper limb rehabilitation in the SCI population, in which an injury at a T1 level or higher affects grasping and reaching functions. The first concrete evidence of benefit from using FES as a therapy was offered in the article published by Popovic et al. (not the coauthor of this chapter) who demonstrated that using the Bionic Glove can improve voluntary upper limb function in individuals with an SCI at a C5–C7 level [48]. In 2005, Mangold et al. [63] also provided anecdotal evidence that for some individuals with SCI who used an FES system as an orthotic system resulted in the recovery of voluntary upper limb function.

Our Contributions to FES Therapy

Over the last two decades, we have developed FES therapies to promote recovery after spinal cord injury and stroke. To conduct our work, we use the Compex Motion neuroprosthesis [55], which was designed specifically for FES therapy, and, depending on what kind of therapy it is used to deliver, it could have from 4 up to 16 stimulation channels. As a result, Compex Motion may be used to produce specific and complex movements (e.g., palmar, lateral, pinch, and lumbrical grasp as well as bipedal locomotion) with a high degree of control. With this technology, we have created FES systems to restore walking in individuals who have suffered a stroke or a spinal cord injury, as well as reaching and/or grasping movements.

With respect to restoration of walking, in 2006 Thrasher et al. [19] tested the hypothesis that direct muscle stimulation would have rehabilitative potential. Five individuals with chronic, incomplete SCI, a population for whom rehabilitation is not expected to produce significant functional changes completed 12–18 weeks of training using the Compex Motion multichannel neuroprosthesis for walking [19]. All of the participants experienced significant improvements in their walking function. Four of them increased their length of stride as well as their stepping frequency resulting in greater walking speeds, while the fifth individual experienced a significant reduction in preferred assistive devices. The results suggest that the multichannel FES-based gait training regime that is directly stimulating muscles instead of using flexor withdrawal reflexes is viable for restoring voluntary gait in incomplete SCI.

More recently, Kapadia et al. [25] compared the short- and long-term effects of a multichannel FES-assisted walking program using a body weight support and treadmill system versus a non-FES exercise program on gait and balance in individuals with chronic, incomplete SCI (level C2–T12). The individuals attended the

training program 3 days a week for 16 weeks in which FES was applied bilaterally to the quadriceps, hamstrings, dorsiflexors, and plantarflexors in the same sequence that they are activated in able-bodied individuals during walking. Spinal cord independent measure (SCIM) mobility subscore improved over time in the participants receiving FES therapy and all other outcomes were similar for both groups. The findings suggest that task-oriented training improves walking ability in individuals with chronic, incomplete SCI. Additional randomized controlled trials need to be conducted to verify if FES-assisted treadmill is superior to aerobic and strength training.

Our research in FES therapy for restoration of upper limb function has yielded important results. In the context of stroke rehabilitation, one of the unique aspects of our work is our focus on restoring reaching and grasping functions in individuals with severe hemiplegia (Fugl-Meyer Assessment $\leq$15) in which the ability to move has been greatly impaired or completely lost, and for whom recovery of motor function after rehabilitation is rare [18]. This is in contrast to the studies mentioned earlier performed by other groups, which included only participants who had reaching and/or grasping functions at least partially preserved. We recently completed randomized control trials [18, 56] to determine the effects of FES therapy for reaching and grasping in severe stroke patients (i.e., Chedoke McMaster Stages of Motor Recovery scores $\leq$2 or Fugl-Meyer Assessment $\leq$15). The findings of these studies suggest that both functions improve with FES therapy and that, in patients with severe hemiplegia, the therapy improved gross motor function but not fine motor movements of the hand. The participants in the FES therapy group experienced median improvement of 24.5 points on Fugl-Meyer Assessment, while the matched control group participants had a median improvement of 0 [18].

In 2006, we conducted the first randomized control trial to assess the impact of FES therapy on grasping after a complete and incomplete traumatic SCI (level C4–C7) [20]. The participants in the study received 40 1-h sessions of either FES therapy or conventional occupational therapy. The study provided clear evidence that participants with both complete and incomplete SCI greatly improved their grasping function following FES therapy as compared to participants who were in the control group.

In a different randomized control trial, we evaluated the effects of FES therapy for restoring grasping in incomplete, traumatic SCI (C3–C7) [21]. All of the participants of the study received conventional occupational therapy for 1 h as described in [20], followed by a 2-h break. After this pause, the subjects received an additional hour of either conventional occupational therapy or FES therapy for grasping. After 40 1-h sessions (5 days a week for 8 weeks), the results revealed a significant and function improvement in participants who received FES therapy [21]. The participants in the FES therapy group experienced median improvement of 12 points on Spinal Cord Independence Measure self-care subscore, while the matched control group participants had a median improvement of 3 points on Spinal Cord Independence Measure self-care subscore [21].

In a long-term follow up study [16], both the FES therapy and conventional therapy groups sustained or improved their hand function compared to their scores at

the time of discharge, suggesting that the dramatic changes in hand function produced by FES therapy persist over time.

We recently reported preliminary evidence demonstrating the potential ability of FES therapy to restore upper limb function (reaching and grasping) in severe chronic pediatric stroke patients [17]. As with adults, rehabilitation of motor function was unsuccessful in this population. There were four participants in that study with hemiplegia, unable to use the affected arm functionally. They received one hour of FES therapy three times per week for 16 weeks (48 sessions in total). All of the participants showed considerable improvements in their upper limb function.

The results that our group achieved with FES therapy for improving reaching and grasping in stroke and SCI individuals, motivated our team to create a product that can be used to deliver this intervention. In 2014, a Canadian company MyndTec Inc. launched first FES system specially developed to deliver FES therapy for restoring upper limb function in stroke and SCI individuals. The product is called MyndMove and it incorporates all stimulation protocols and technology our team has developed in this field in the last two decades. MyndMove offers 17 FES-based interventions for stroke individuals and 13 FES-based interventions for SCI individuals.

In conclusion, there is strong evidence to support the use of FES therapy as an effective tool for retraining of walking, reaching, and grasping functions after a stroke or an SCI. In the remainder of the chapter, we will provide possible explanations to the effectiveness of FES therapy in restoring motor function.

Selected Processes in the Healthy Central Nervous System Pertinent to Neurorecovery following an Injury to the Central Nervous System

Neuroplasticity and the Healthy Adult Brain

The FES therapy program is designed to optimize the neuroplastic potential of the adult brain. It is well established that adult neural stem cells (NSCs) and neural progenitor cells (NPCs) are present throughout the CNS [64–67]. However, they are more densely distributed in two particular subregions of the adult brain, the subventricular zone and the hippocampal subgranular zone [64]. Regardless of their locations, the precursors possess the capacity for inexhaustible self-renewal and pluripotency (i.e., they can differentiate into a wide variety of neurons, astrocytes, and/or oligodendrocytes) [68]. Research has shown that adult neurogenesis is highly regulated. In 1998, Wang et al. [69] demonstrated that self-renewal and regeneration normally occur in the adult brain through highly specific and targeted neuronal apoptosis. The signals that guide the establishment and maintenance of neuronal diversity and connectivity during development also play a pivotal role during differentiation and integration of new adult-born neurons within the neurogenic niches in the adult brain [69]. Differentiation of adult-born neurons (i.e., commitment to a

particular neuronal identity) is guided by endogenous and exogenous stimuli, genetic, and epigenetic factors [70]. During differentiation, the immature neurons are constantly learning and adapting to their microenvironment. These experiences and learnt lessons are unique to each immature neuron. At maturation, these neurons will become integrated (synaptogenesis) into the preexisting neural network(s). This permits the neural network to preserve old memories and store new memories. The addition of new memories to the neural network enables it to adapt to its constantly changing environment and to maintain homeostasis in a "hostile" microenvironment [71–73].

The Healthy Neurological System and Exercise

Adult neurogenesis has been shown to be acutely responsive to changes in its microenvironment. These changes may result from external or internal stimuli as well as from genetic and epigenetic factors [68]. One of the most studied influences on adult neurogenesis is the role of exercise in learning and memory creation. It is well established that exercise increases neurogenesis in the healthy brain of rodents and humans [74–78]. In 2010, Kobilo reported that adult neurogenesis in the mouse hippocampus was enhanced by voluntary exercise in a running wheel [70, 74]. The onset of the effect in the hippocampus was rapid. Running induced cell proliferation in adult mice. It peaked after 3 days of running and was significantly enhanced at 10 days. After 32 days of running, the proliferative effect returned to baseline, but the number of new neurons continued to increase. More importantly, exercise was shown to enhance the maturation of the newborn neurons. Enhancement of hippocampal neurogenesis by running is a robust phenomenon that has been replicated by many different laboratories [70, 74–78]. Exercise-induced increase in neurogenesis is associated with enhanced hippocampal synaptic plasticity, more specifically, long-term potentiation (LTP) [70]. Becker and Wojtowicz [79, 80] reported that new neurons generally lack inhibition and have superior ability to express LTP. Both of these properties make new neurons suitable for synaptic integration via spatial and temporal summation of afferent synaptic inputs [80] and ideal for creating new memories [79–81].

The Injured Neurological System, Neurogenesis, and Exercise

What blocks the brain from recovering functional motor and sensory activity following a catastrophic injury or insult? We know that regardless of age (infant, child, young adult, adult, or aging individual), the clinical outcome is related to the severity, etiology, and location of the "lesion" [82, 83]. Why are the NSCs and the NPCs unable to comprehend the severity of the damage to the brain and mount an adequate response?

What we do know is that the neural networks possess all of the necessary equipment to ultimately recover from serious injury or neurodegenerative disease. What remains a mystery is why the system appears to become terminally disabled by these insults. Varying degrees (partial) of functional motor and sensory activity are recoverable following a catastrophic CNS with the assistance of standard rehabilitation practices. Standard rehabilitation practices include a prescription of or assistance with specific exercises, manual therapy, education, manipulation, and other interventions. Rehabilitation programs are designed to optimize the benefits of exercise with respect to neurogenesis, learning, and memory. Yet, despite our valiant efforts, we are unable to achieve complete or incomplete functional motor and sensory recovery for patients who have sustained a catastrophic neurological injury or who have a neurodegenerative disease. Why?

We hypothesize that several unfavorable conditions may exist in both the injured neural tissue and in tissue affected by a neurodegenerative disease. First, the rate at which the damage occurred may result in an insufficient number of available precursor cells to bring about functional recovery. Second, the balance between neurogenesis and gliogenesis may be tipped in favor of gliogenesis. The differentiation fates of endogenous precursors may be too limited to permit adequate differentiation fates of the endogenous precursors and too limited to allow their integration into varied portions of the brain. Third, the potential challenge is that it could be difficult to provide the precise combination and sequence of molecular signals necessary to induce endogenous precursors to proliferate efficiently and differentiate precisely into appropriate types of neurons deep in the brain. It is well documented that the timing of neurogenesis during development is highly correlated with neuronal laminar position and subsequent connectivity [71]. At this time, it is unknown if the same developmental sequence of events followed by neuroblasts in the developing brain are followed by NPCs and NSCs.

There is also another aspect to this challenge. Following CNS injury, body parts that were previously controlled by the injured part of the CNS are left affected or paralyzed due to the injury. If the limb or body part is mildly affected by the injury, and the patient is able to use it partially, the individual can be engaged in repetitive exercise treatments that eventually can help partially or completely restore the function of the affected limb or body part. This concept has been well demonstrated with the constraint-induced movement therapy (CIMT) [84].

However, when the neurological patient does not have any residual motor function, then one cannot deploy repetitive exercises/therapies, and the prospects of motor function recovery are reduced almost to zero. This can be explained by the loss or compromised control of the muscles by the responsible part of the CNS due to severe injury. Over time, the neuromuscular system associated with a particular limb drifts into two possible extreme "modes of operation," flaccid paralysis or paralysis in which many of the limb muscles are contracted most of the time. Or the neuromuscular system assumes a "mode of operation" that is between these two possible extremes. No matter in which state the system "settles in" the patient is typically not able to voluntarily activate the muscles of interest and this with time results in "learned nonuse" of the affected limb or body part [84]. Once the patients

reach the state of "learned nonuse" it is extremely difficult to help them relearn the affected motor task(s). Also, as the process of "learned nonuse" progresses, the remaining intact parts of the CNS that were before engaged in performing a desired motor task are with time "hijacked" by other motor, sensory, or cognitive tasks and become engaged in performing these new tasks. This process is known as neuroplasticity; more specifically, this is a form of neuroplasticity which is not necessarily desirable, especially in neurologic patients, as the "memory" of performing the tasks of interest slowly "fades" away with time.

The combination of cell, tissue, and circuit level challenges, as discussed above, is caused by the CNS injury and ultimately results in "learned nonuse" that frequently interferes with functional motor recovery. If any of these challenges could be addressed effectively, and, preferably, if few of them could be addressed simultaneously and successfully, one would be able to potentially help restore voluntary function in severe neurologic patients.

The Injured Neurological System and Functional Electrical Stimulation Therapy

How does FES therapy provide individuals with a neurological injury or neurodegenerative disease with the opportunity to recover sustained functional motor activity, while electrical stimulation does not? FES therapy is not simply the application of an electrical stimulus to the paralyzed muscle(s). We believe that FES therapy may be the ideal augmentative rehabilitation intervention. In addition to the physical rehabilitation benefits, recent studies suggest that FES therapy may facilitate or augment the repair of the injured nervous system. It has the potential to functionally reconstruct the damaged neural circuits through its potential ability to promote the self-regeneration capacity of the CNS by promoting:

1. Robust regeneration and replenishment of neural cells.
2. Robust regeneration and repair (myelination) of axons.
3. Providing the necessary rhythmical and spatiotemporally organized efferent and afferent inputs to ensure that the synaptic connections are organized and operate according to the somatotopic maps and designated functions.

FES therapy uses electrical stimulation to guide the NPCs and NCSs to the site of the lesion. These electrical activity patterns influence a variety of developmental processes during corticogenesis, such as neurogenesis, apoptosis, neuronal migration, differentiation into a variety of different neurons, and network formation [85–88]. The information carried in these signals is critical not only during the initial organization of the nervous system but is perhaps even more critical during adulthood [89]. Later, experience-dependent, use-driven adaptations are encoded by the spatiotemporal pattern of sensory processing and intrinsic neural activity that lead to declarative learning and the acquisition of procedural skills [89]. Both forms of neural activity shape synaptic development [89].

Numerous studies have explored the role of electric fields in the CNS. Physiological direct current electric fields (dcEFs) play important roles during development and in tissue repair [86, 90–94] and have been shown to cathodally direct the turning of growth cones during axon elongation [91, 95]. Babona-Pilipos et al. [90, 94] have demonstrated that clonally derived pure populations of adult SE-derived NPCs exhibit rapid and directed galvanotaxis toward the cathode of a dcEF. This phenomenon is unique to undifferentiated NPCs. By inducing NPCs, maturation into differentiated phenotypes is associated with a loss of electrically induced migratory capacity. Thus the data indicate that externally applied dcEFs can stimulate and guide the migration of undifferentiated SE-derived NPCs, but not that of NPCs induced to differentiate into mature neural phenotypes. Similar studies have been performed on the NPCs that reside in the periventricular lining of the central canal. Therefore, FES therapy may assist in the self-repair and regeneration process by selectively increasing the volume of the neural cells at the site of the lesion and providing critical functional spatiotemporal information during their differentiation and maturation phases.

Our research has definitely demonstrated that FES therapy promotes increased neural activity below the level of injury in incomplete spinal cord injury rats [84, 96]. Experiments conducted by our team using animal models have shown that the FES therapy promotes rewiring of the neuronal circuitry below the level of spinal cord injury and that it also promotes propagation of the afferent signals over the site of injury to the somatosensory cortex [96]. These changes in the CNS activity following short-term FES therapy for walking (therapy was delivered to incomplete spinal cord injury rats—dose 15 min per session, three times per day, for 7 days) were not observed in the control group that was implanted with the FES system, but did not receive the FES therapy.

We have clearly established that it is critical that FES is administered while the patient is trying to perform the task. By doing that we are essentially generating proper muscle activation and proper sequence of muscle activities needed to carry out a desired task. This in turn produces popper muscle tension that is essential for producing needed afferent signals. Only muscles that are contracted with the proper level of intensity generate adequate afferent signals produced by muscle spindles, Golgi tendons, and other sensory receptors. If the muscles are not active, and they do not move along the desired "muscle contraction profile" they do not produce relevant afferent signals. This is why passive limb movements produced manually or using robotic systems are not providing sufficient afferent feedback needed for retraining motor tasks. It is this volley of afferent input combined with motor task planning and proper efferent input that are essential for the retraining of the injured CNS. More specifically, our clinical studies suggest that if a neurologic patient who attempts to execute a motor task is assisted with the FES therapy to carry out that task, he/she is effectively voluntarily generating the motor command. FES therapy is then providing both efferent input and afferent feedback (system's output), indicating that the command was executed properly, successfully, and in physiologically correct manner. We hypothesize that by providing both the "command input" and "system's output" to the CNS repetitively over enough time, this type of

treatment facilitates functional reorganization within the sensorimotor network. We believe that the combination of performing diverse and meaningful tasks with high repetition and subject's persistent active engagement (i.e., subject has to devote 100 % of his/her attention to the tasks performed) is playing a critical role in retraining voluntary motor functions. These strategies are fully in tune with the recent findings in the field of neuroplasticity [97] and suggest that the proposed FES therapy is potentially very effective method that can be used to retrain the neuromuscular system.

The CNS is a distributed system. This essentially means that even though some parts of the CNS are "more" responsible for performing a particular task, other parts of the system are also engaged. Therefore, following the injury to the CNS a part of the subsystem that is mainly responsible for carrying out a particular task may be damaged, but the other "less engaged" subsystems may remain intact and receptive to retraining. Currently, scientists have focused their research at exploring how the cortex can be "plastically" changed to accommodate these changes and retraining. It is our belief that phylogenetically older brain structures, such as the brain stem, may also have a capacity to relearn some motor tasks. The fact that severe stroke patients following FES therapy for reaching and grasping often relearn how to voluntarily grasp and release objects but are unable to relearn fine finger motor tasks may suggest that phylogenetically older brain structures have been engaged in the process of reaching and grasping task relearning [18]. In SCI patients, relearning fine finger motor tasks may not be such a challenge as it is in severe stroke patients. This suggests that in SCI patients the neuronal recovery occurs at the level of spinal cord and it allows more complex, cortical (supraspinal) commands to flow to the spinal cord below the level of lesion, allowing patient to relearn fine motor tasks.

We recently completed a clinical study with a chronic severe stroke patient [23]. We observed that even if the FES protocol is not a 100 % accurate representation of the actual upper limb movement and it is not engaging all relevant muscles, but only most prominent ones, the CNS will fast realize which muscles also need to be activated, in addition to the stimulated ones, to generate proper hand or arm movement. As a result, after 5–10 sessions the CNS itself will start engaging all relevant muscles in order to perform the task of interest. We were able to observe this finding by measuring voluntary EMG activities on both stimulated and nonstimulated muscles. Not only did the CNS demonstrate ability to regain voluntary control over the stimulated muscles following the FES therapy, but it was also able to regain voluntary control over the muscles that were not stimulated but are important for the correct performance of the desired task. We interpret this finding in the following manner. The volleys of the efferent input and afferent feedback indicate to the CNS that it is asked to perform a task which it until recently was performing all the time. The CNS then "recognizes" the tasks and voluntarily attempts to perform it. As its ability to recruit stimulated muscles increases the CNS automatically starts engaging both stimulated and other relevant muscles needed to carry out the task. Essentially, the "memory" of the neuromuscular systems is being refreshed and as this memory is becoming more and more engaged the system starts engaging all muscles of relevance in physiologically correct manner in order to carry out the desired task.

Finally, in addition to the clinically relevant and meaningful improvements in voluntary motor function we have achieved using FES therapy, we have also observed a myriad of other clinical benefits that FES therapy offers. Many of our patents experienced immediate reduction in spasticity and muscle tone, which later persisted following therapy completion. Others reported reduced pain, better posture, improved bladder and bowel function (especially patients who took part in the FES therapy for walking), and improved muscle and skin condition. All stroke patients reported that shoulder subluxation ceased to be a problem after 10+ therapy sessions. Also, one chronic stroke subject (>2 years poststroke), who received FES therapy for reaching and grasping, experienced dramatic improvement in speech following 20 treatments. As this is not the topic of this article, we will discuss these findings at another more opportune time.

Conclusion

We propose that the FES therapy restores functional motor activity by supporting the functional reconstruction and reorganization of the neural circuits in the CNS. The FES therapy does that by (1) enhancing neurogenesis (the recruitment, regeneration, and differentiation of neural progenitor stem cells) at lesion; (2) spatially and topographically organizes synaptogenesis (axonal regeneration and collateral sprouting) and remyelination; (3) reactivates the "memory" in the neuromuscular system; (4) helps create new neural networks within the preserved parts of the CNS that will substitute the function of the damaged part of the nervous system to allow it to control and execute desired motor functions; (5) by repetitively providing proper efferent and afferent input, it helps create and retrain the neural networks described in (4); and (6) maintains the integrity of the neuromuscular system.

The clinical results achieved to date in restoring voluntary reaching and grasping function in severe stroke and SCI individuals suggest that these improvements are dramatic and clinically relevant and that the FES therapy has to be taken into serious consideration as the potential new best practice for restoring upper limb function, at least in these two patient populations. As for the walking therapy, more rigorous randomized control trials are needed before we can say with confidence that the FES therapy is effective in restoring voluntary locomotion function in stroke and SCI individuals, although the initial findings are encouraging.

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Chapter 26
Deep Brain Stimulation for Neuropsychiatric Disorders

Ausaf A. Bari, Nicolas Kon Kam King, Nir Lipsman, and Andres M. Lozano

Abstract Neuropsychiatric disorders that are refractory to best medical management continue to pose a significant challenge. Advances in molecular neuroscience and neuroimaging have started to reveal how dysfunction in specific limbic networks mediates these disorders. This knowledge, along with concurrent advances in neurosurgical techniques, has lead to the increasing use of deep brain stimulation (DBS) for the treatment of neuropsychiatric disorders. Here we review the role of DBS for obsessive-compulsive disorder (OCD), depression, addiction, post-traumatic stress disorder (PTSD), and anorexia nervosa (AN). We emphasize the use of translational techniques such as neuroimaging, molecular neuroscience, and animal models in guiding and evaluating the use of DBS for these challenging disorders.

Keywords Deep brain stimulation (DBS) • Obsessive-compulsive disorder (OCD) • Depression • Addiction • Post-traumatic stress disorder (PTSD) • Anorexia nervosa (AN) • Limbic system • Neuropsychiatric disorders

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Introduction

Deep brain stimulation (DBS) is a neurosurgical treatment that delivers current through electrodes surgically implanted into specific brain regions. In the past 20 years, DBS has emerged as a successful treatment for movement disorders such as Parkinson's disease, essential tremor, and dystonia. The entire DBS system consists of intracerebral electrodes connected to a small programmable battery implanted under the skin. Current delivery through these electrodes results in the reversible and adjustable modulation of pathological neuronal networks underlying specific neurological diseases. In patients with movement disorders such as Parkinson's disease, DBS results in significant improvement in symptoms by directly modulating the abnormal activity in the motor circuit. To date, DBS has been used to treat over 100,000 patients worldwide with the major advantages of the procedure being reversibility and programmability.

The success of DBS in movement disorders has prompted the medical community to investigate whether the benefits of DBS may be used to treat psychiatric disorders refractory to conventional therapy. Psychiatry has evolved from thinking about the brain as a black box toward the understanding that psychiatric disorders are caused by changes in specific neuronal networks that can potentially be modulated by DBS. The health and socioeconomic cost of mental illness to society are substantial. Although, effective pharmacological and behavioral therapies continue to evolve, there is a significant proportion of patients that is refractory to conventional treatment and may benefit from DBS.

In the past, the surgical treatment of mental illness relied mainly on ablative procedures in which permanent lesions were created in specific brain areas thought to be involved. Initial attempts were crude by today's standards and often resulted in large, nonselective lesions such as the frontal lobotomy introduced by Egas Moniz in 1936 [1]. Eventually, with the advent of stereotactic targeting, the frontal lobotomy gave way to more selective lesions such as anterior cingulotomy, subcaudate tractotomy, anterior capsulotomy, and limbic leucotomy [2, 3] (Fig. 26.1). Additional advances in stereotactic frames and radiosurgery allowed neurosurgeons to produce lesions in a more precise and noninvasive manner. The major disadvantage of using lesions to treat psychiatric disorders is their permanent nature and the inability to modulate the treatment in response to changes in a patient's clinical response. The latter is arguably even more important in psychiatric disorders given the variability between patients and within a given patient over time. In contrast, DBS overcomes these limitations and because of its anatomical specificity, reversibility, and programmability, has been increasingly studied and refined over the past 10 years as an effective treatment for neuropsychiatric disorders.

The term "neuropsychiatric disorder" is broad and may encompass any neurological disorder with a psychiatric component. Here we will focus on disorders that are primarily psychiatric and review the role of DBS in the following five conditions: obsessive-compulsive disorder (OCD), treatment-resistant depression (TRD),

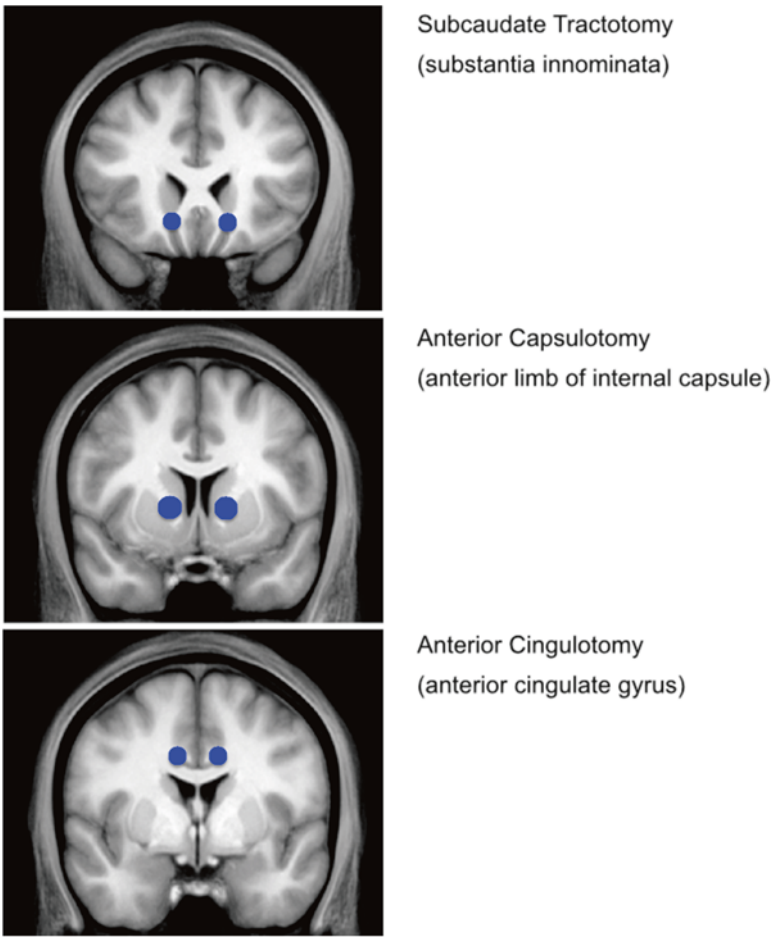

Fig. 26.1 Lesion locations for subcaudate tractotomy, anterior capsulotomy and anterior cingulotomy. *Blue circles* indicate anatomical location of the lesioned fiber tracts superimposed on an MRI representation (coronal sections).

addiction, post-traumatic stress disorder (PTSD), and anorexia nervosa (AN). However, the list of neuropsychiatric conditions for which DBS has been proposed and studied is much larger and beyond the scope of this review. For a discussion of the emerging role of DBS in other neuropsychiatric disorders such as Schizophrenia, Tourette's syndrome, and Alzheimer's disease, the reader is directed to a prior review by our group (Lozano and Lipsman 2013) [4]. Where applicable, we will emphasize the role of translational techniques such as animal models, biomolecular techniques, and structural and functional neuroimaging in the application of DBS to these disorders (Fig. 26.2).

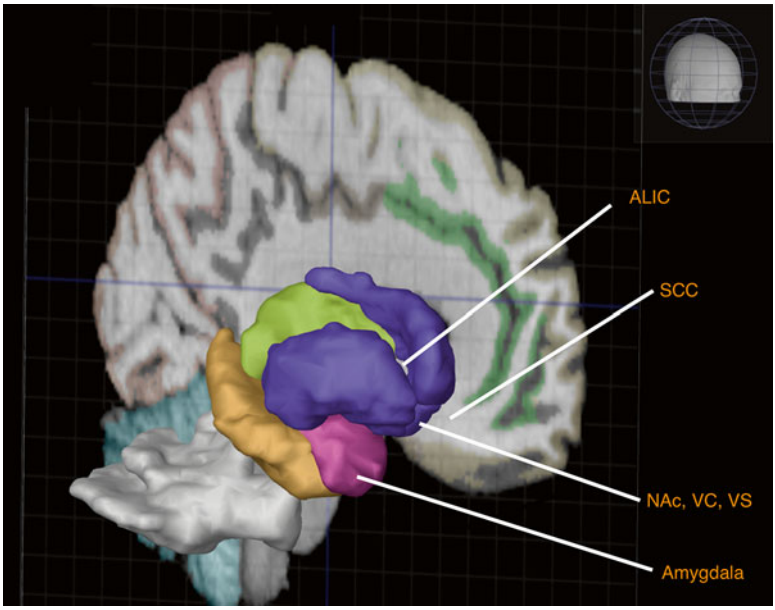

Fig. 26.2 Anatomical view of common limbic DBS targets. *ALIC* Anterior limb of the internal capsule, *SCC* Subgenual cingulate, *NAc* Nucleus accumbens, *VC* Ventral capsule, *VS* Ventral striatum.

Limbic System

The brain structures that make up the limbic system are thought to form the emotional core of the brain. Key components of the limbic system include the amygdala, cingulate gyrus, hippocampus, insula, medial prefrontal cortex (mPFC), nucleus accumbens (NAc), orbitofrontal cortex (OFC), ventral striatum (VS), and ventral tegmental area (VTA) [5]. Pathological changes within these areas have been associated with nearly every neuropsychiatric condition, including mood, anxiety, and psychotic disorders. Through its connectivity with the rest of the brain, the limbic system integrates memory, emotion, reward, motivation, and goal-directed behavior to maximize the survival of the organism.Mental illness results from functional or structural changes in these networks. The efficacy of DBS lies in its ability to modulate the specific pathological networks underlying each disorder. The challenge of applying DBS to mental illness lies in identifying the most effective targets, selecting the appropriate patients, optimizing stimulation parameters, and characterizing the structural and functional heterogeneity between patients.

Obsessive-Compulsive Disorder

OCD is characterized by unwanted and recurrent intrusive thoughts (obsessions) and repetitive behaviors (compulsions) that are aimed at relieving the distress caused by the obsessions [6]. Up to 30 % of patients with OCD are resistant to traditional behavioral and pharmacological treatment [7]. Prior to the advent of DBS, the surgical treatment for refractory OCD involved the creation of lesions within several limbic structures. While early attempts were crude and nonspecific, the advent of stereotactic techniques in the mid-twentieth century lead to the creation of focal and more precise lesions. In 1949, the French neurosurgeon Talairach developed a stereotactic method to thermally ablate fibers in the anterior limb of the internal capsule (ALIC) [8]. This "anterior capsulotomy" created a disconnection between the OFC and subcortical limbic areas such as the NAc, amygdala, and thalamus. Subsequent lesioning procedures included the subcaudate tractotomy and anterior cingulotomy. Subcaudate tractotomy, a procedure developed in London in 1965, involves the stereotactic placement of radioactive ytterbium-90 to ablate the substantia innominata [9]. In 1967, Thomas Ballantine introduced the anterior cingulotomy in which stereotactic air ventriculography was used to target the anterior cingulate region with thermal ablation [10]. A fourth procedure, the limbic leucotomy, was the result of combining the anterior cingulotomy with the subcaudate tractotomy and was popularized in the UK [8] (Fig. 26.1). Ultimately, Larks Leksell refined Talaraich's ablation of the ALIC by introducing the Gamma Knife capsulotomy. This had the advantage of allowing lesions to be made noninvasively without surgically opening the skull [8].

The clinical efficacy of these lesions laid the conceptual groundwork that would eventually be used to guide DBS trials for OCD on the basis that DBS may work by mimicking the effects of a lesion. The first clinical trial of DBS for OCD consisted of bilateral DBS of the ALIC in four patients with refractory OCD. The trial was successful given that three out of the four patients showed benefit [11]. Follow-up studies of ALIC DBS showed a 50% response rate as defined by an improvement of at least 35% on the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) [12]. Based on the results of these pilot studies, OCD became the first psychiatric disorder to be approved for DBS in North America on a humanitarian device exemption basis.

Other DBS targets for OCD that have shown promising results include the ventral capsule/ventral striatum (VC/VS), NAc, inferior thalamic peduncle (ITP), and subthalamic nucleus (STN) [13–18]. The efficacy of stimulation of such diverse targets supports the idea that OCD is a disorder resulting from pathological changes in a neuronal network that links affective, limbic areas with motor areas that result in behavioral output. Similarly, the involvement of the STN in a psychiatric disorder makes sense in the context of a broader network linking the limbic and motor systems. In addition, the STN itself consists of motor, associative, and limbic subregions and pathological involvement of the latter may underlie its role in OCD [19]. The identification of the most efficacious target, or set of targets for OCD,

will require further clinical trials with a larger number of patients, combined with insights derived from DBS studies in preclinical animal models.

Animal studies do not always precede human clinical trials, particularly in the neuropsychiatric literature.. As discussed above, DBS for OCD evolved from the lesioning experience in patients rather than being derived initially from animal studies. A possible explanation may be the difficulty of studying psychiatric phenomena in animals. For example, while *compulsions* may be modeled in animals by inducing repetitive behaviors, *obsessions* are more difficult to model. Indeed, a general critique of animal models for psychiatric disorders is their inability to accurately measure the internal affective states that are integral to the characterization of these disorders. Despite these limitations, animal models of compulsive behavior may be used as surrogates of OCD in order to investigate new DBS targets, optimize stimulation parameters, characterize the timeframe for a clinical response, and to evaluate advances in DBS technology [20].

OCD research has also benefited from optogenetics, a technology that has recently increased our understanding of the mechanisms underlying DBS for both motor and psychiatric disorders. Optogenetics involves the targeted expression of light-sensitive channelrhodopsin proteins in selected neuronal cell types in awake animals. Depending on the specific type of channelrhodopsin expressed, optical stimulation can result in hyper- or depolarization of the target neurons. This technique has allowed researchers to directly test hypotheses regarding the causal role of individual neuronal types in different disease processes [21]. Optogenetics also allows simultaneous *in vivo* recording during stimulation, avoiding the stimulus artifact often associated with DBS [22]. Optogenetic stimulation of the OFC and VC/VS has been shown to improve compulsive behavior in a mouse model of OCD [23, 24]. Although optogenetics is currently limited to animal studies, it may help elucidate the neural circuitry underlying neuropsychiatric disorders in general, identify novel targets for stimulation, and help elucidate the mechanisms underlying the therapeutic effects of DBS. Although currently impossible, we can speculate a future in which advancements in optogenetics could be used to replace DBS electrodes with light-emitting "optrodes." This would improve the "resolution" of DBS by targeting specific cell types rather than nonspecifically modulating entire brain regions.

In addition to animal studies, functional and structural neuroimaging in human subjects has been essential in characterizing the brain networks underlying neuropsychiatric disorders. For example, diffusion tension imaging (DTI) has been used to probe the structural connectivity of the limbic networks implicated in OCD [25]. DTI tractography is a technique that uses MRI sequences sensitive to the diffusion of water molecules along axons in order to noninvasively estimate the connectivity between different brain regions. For example, tractography has been used to show the relative spatial organization of orbitofrontal fiber pathways involved in subcaudate tractotomy [26]. The use of preoperative DTI has also been proposed to improve DBS targeting based on the patient's specific structural connectivity [27].

The use of DTI and functional MRI (fMRI) to study healthy subjects has allowed investigators to "virtually dissect" the networks involved in normal limbic processing and compare them to patients with the disease. Data from such studies can subsequently be used to discover novel, more selective targets for DBS [28].

For example, using fMRI, DBS of NAc for OCD has been shown to normalize NAc activity by reducing the functional connectivity between the NAc and the prefrontal cortex, restoring disease-related brain networks in OCD to the state found in healthy subjects [29].

Depression

Major depression is the most common psychiatric disorder and a major source of adult disability [30]. Approximately, 10–20% of patients with major depression are refractory to treatment including antidepressants, psychotherapy, and ECT. Evidence has shown that major depression, like other neuropsychiatric conditions, is a systems-level disorder involving multiple brain regions, neurotransmitters, and molecular mediators [31]. Over the past 10 years, DBS of several limbic areas has been shown to improve symptoms of depression. To date, these include the subgenual cingulate cortex (SCC), NAc, VC/VS, ALIC, the lateral habenula (LH), ITP, and the medial forebrain bundle (MFB) [31–36].

Our center has focused on the SCC as DBS target for patients with TRD (Fig. 26.3). The SCC, which includes Brodmann Area 25, is a subregion of the anterior cingulate gyrus that lies ventral to the corpus callosum (See review by Hamani et al. 2011) [37]. Functional imaging played a central role in identifying this region as a target for DBS. PET imaging revealed an increase in activity in the SCC in patients with major depression which was reversed with antidepressant therapy [38]. In addition, there was an increase in glucose metabolism in this region when subjects were asked to recall sad autobiographical events [39]. Based on these studies, an open-label trial of high frequency bilateral DBS of the SCC was approved in six patients with severe refractory depression. Six months after surgery, four out of the six patients had either a sustained clinical response or remission as defined by at least a 50% reduction on the Hamilton Depression Rating Scale (HDRS) [31].

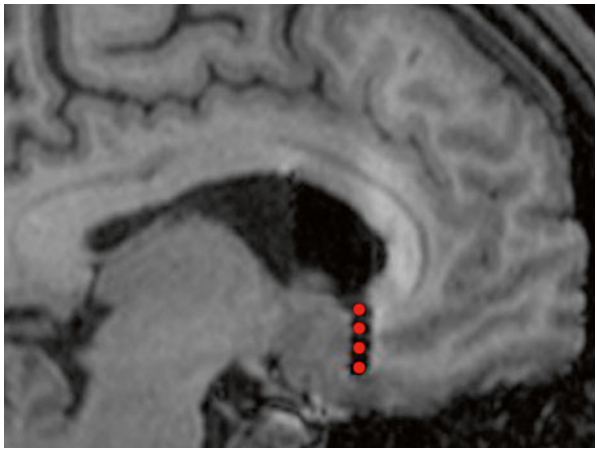

Fig. 26.3 Representative sagittal postoperative MRI of a DBS electrode placed in the SCC (Area 25) for depression or anorexia nervosa. *Red circles* indicate location of electrode contacts relative to white matter pathways of the medial orbitofrontal region and cingulum bundle.

Furthermore, clinical response was associated with a decrease in glucose metabolism in the SCC [31]. Given the promising results from this pilot study, DBS of the SCC was expanded to include 20 patients with a 75% response at 3 years follow-up [40]. However, a recent multicenter, randomized clinical trial of SCC DBS for TRD was discontinued based on the results of a futility analysis (unpublished results of the BROADEN study). Future studies with modifications to the inclusion criteria and stimulation parameters may be necessary in order to observe a clinical benefit from SCC DBS [41].

As in the case of OCD, DTI and optogenetics are playing a greater role in studying the mechanisms underlying depression. Studies using DTI in conjunction with electrical field simulation (EFS) suggest that DBS of the NAc, SCC, ALIC, and VC/VS can all result in activation of the MFB [42]. The MFB may constitute the final common pathway by which DBS of such disparate structures can result in clinical efficacy. Probabilistic tractography has been used to characterize the differences between patients in their response to DBS for depression. Probabilistic tractography in 16 patients that underwent bilateral SCC DBS for TRD revealed significant differences in structural connectivity between responders and nonresponders. Specifically, it was found that three different fiber pathways in this region are likely to mediate the clinical response to DBS: the forceps minor, cingulum bundle, and the medial branch of the uncinate fasciculus [43]. Adjusting the stimulation such that all three bundles were covered resulted in the conversion of nonresponders to responders. In the future, prospective use of DTI and fMRI may allow patient-specific targeting leading to more efficacious DBS. This is particularly pertinent for neuropsychiatric disorders given that there may be significant network heterogeneity between patients refuting a "once size fits all" approach to DBS for these conditions.

Another major challenge in the use of DBS for depression is that typically the clinical response is delayed and may not be evident until several months after the start of DBS. Unlike in movement disorders such as Parkinson's disease or essential tremor, it is not clear that an acute clinical response can be used to verify the proper position of the DBS electrode. Furthermore, without an acute clinical effect, there is little information to guide the clinician as to what initial stimulation parameters should be used in programming the system for a given patient. Despite the absence of an observable clinical effect, functional neuroimaging may help identify "biomarkers" that may be used as surrogates in the absence of an acute response. However, while fMRI provides high spatial resolution, it suffers from low temporal resolution and is currently not approved for use in patients with DBS hardware. On the other hand, magnetoencephalography (MEG) is completely noninvasive, has a temporal resolution on the order of milliseconds, and can be used safely in patients with DBS. The application of novel techniques such as MEG may reveal the acute neurophysiological response to DBS that precedes an actual clinical response. The identification of imaging biomarkers may ultimately help improve placement of electrodes, as well as identify the appropriate programming parameters in a patient-specific manner.

Similar to OCD, DBS for depression was guided by human ablation studies and neuroimaging in human subjects rather than preclinical animal models. Once human

trials were already underway, animal models were used to further understand the mechanisms underlying the efficacy of DBS. For example, high frequency stimulation of the rodent vmPFC (an area homologous to the SCC in humans) and stimulation of the NAc result in antidepressant-like effects as measured by the forced swim test (FST) and the chronic unpredictable stress (CUS) model of depression [44–46]. Preclinical use of animal models will help guide further research on DBS for depression by characterizing new targets, optimizing stimulation parameters and by clarifying the expected time-frame for a clinical response [20].

Addiction

Drug addiction is a major global health concern and accounts for approximately 25% of all deaths in industrialized countries [47]. The list of abused substances includes but is not limited to alcohol, nicotine, opioids, amphetamines, and cocaine. Despite the high relapse rate and lack of a definitive cure for drug addiction, there have been relatively few clinical studies evaluating DBS as a treatment.

Lesion studies have shown that ablation of both the NAc and cingulate gyrus has beneficial effects in patients with drug dependence. In many of these cases, the lesions were performed for other indications such as OCD, depression, or intractable pain and their beneficial effect on comorbid drug addiction was noted only in retrospect. The substances studied included nicotine, alcohol, and opioids. However, few studies have studied lesions in a prospective manner. A series of prospective uncontrolled studies of bilateral ablation of the NAc for opioid addiction reported a reduction in dependence and craving and a 47% abstinence rate at 5-year follow-up [48–50].

As in the case of lesions, the majority of the clinical evidence supporting a role for DBS in addiction comes from patients who underwent DBS for other disorders with an incidental improvement in their comorbid substance abuse. These include patients who had undergone NAc DBS for OCD or STN DBS for Parkinson's disease and were subsequently found to have significant reductions in alcohol consumption or smoking [51–53]. These unexpected findings were subsequently replicated in prospective clinical trials using the NAc as the DBS. In the first of these trials, high frequency DBS of the bilateral NAc was performed in three patients with long-term treatment-resistant alcohol dependence. The authors reported complete reduction in craving in all patients and remission at 1 year follow-up in two of the patients [54,55]. Subsequent trials in small groups of patients have also supported the efficacy of NAc DBS for alcohol and heroin addiction [56–59]. These studies suggest that high-frequency DBS of the NAc may be a valid option in patients with drug addiction who have failed traditional medical management.

DBS has been studied in several animal models of drug addiction. For example, high frequency stimulation of the NAc, STN, lateral habenula, mPFC, and lateral hypothalamus has been found to be effective in reducing drug-seeking behavior in animals [20]. Addiction spans a range of behavior and emotions including the acute hedonic reaction, withdrawal, craving, drug seeking, and relapse. Specific animal

models have been developed to study each of these phenomena. Animal models of addiction have been shown to have good predictive validity and can be used to guide further DBS trials in patients and to evaluate how DBS affects the separate behavioral and affective components of addiction. In addition, optogenetics has been used in these animal models to deconstruct, at the cellular and synaptic levels, the limbic reward pathways involved in drug addiction [60,61]. Taken together, these studies have reinforced the important role of the dopaminergic pathways from the VTA to the NAc and mPFC. The further use of optogenetics and animal models will help determine the most effective targets and help optimize stimulation parameters for maximum clinical results.

As for OCD and depression, a major drawback of using animal models to study addiction lies in the inability to directly measure the affective states that play a central role in this disorder. To circumvent this problem, the affective or emotional component of substance abuse can be studied noninvasively in human subjects with the help of structural and functional neuroimaging such as DTI tractography, fMRI, and MEG. These techniques have been used to improve the clinical efficacy of DBS by guiding electrodes away from nearby unrelated fiber pathways to reduce unwanted side effects and to improve the accuracy of targeting of the intended structure [27]. The MFB constitutes a final common pathway of connectivity between the VTA, mPFC, and NAc, areas known to be involved in reward and addiction. Given the fact that the precise location of the MFB may vary between patients, preoperative tractography may allow patient-specific mapping of this and other potential DBS targets [42]. Finally, fMRI and MEG can also be used to study connectivity and activation of the brain areas during rest and while performing specific reward tasks. The large-scale application of these imaging techniques to build population-level datasets is now underway. For example, the Human Connectome Project (HCP) contains a repository of structural MRI, DTI, resting-state fMRI, task fMRI and MEG data obtained in hundreds of healthy subjects [28,62]. The increased statistical power of these large population-based datasets will help us to identify the differences in brain connectivity between patients and healthy subjects, and help identify areas that are involved in specific psychiatric disorders.

PTSD and Anxiety

PTSD is an anxiety disorder that results following a traumatic event that has threatened the life or integrity of the individual. PTSD can affect the perceptual, cognitive, affective, and physiological domains. It is characterized by hyperarousal, intrusive recollection of traumatic memories, and persistent avoidance of stimuli associated with the traumatic event [63]. The estimated lifetime prevalence of PTSD in the US is approximately 7% [64,65]. Thirty percent of patients still suffer from PTSD ten years after the inciting traumatic event despite best medical therapy [63].

Functional neuroimaging in combat veterans has shown that the amygdala plays a central role in the development of PTSD. PTSD patients subjected to provocative

stimuli show increased activity in the amygdala on both fMRI and PET [66,67]. On PET imaging, the intensity of activation of the amygdala correlates with the severity of PTSD symptoms [66,68]. In another study, 40% of veterans with traumatic brain injury resulting from combat developed PTSD, but only when the amygdala itself was spared. On the other hand, in the veterans whose injury included the amygdala, none developed PTSD [69]. Indeed, the amygdala may be necessary for encoding and retrieval of memories associated with traumatic events, a hypothesis supported by its extensive connectivity to other limbic areas such as the NAc, mPFC, and hippocampus [70]. These findings have lead to the hypothesis that inactivation of the amygdala with high-frequency DBS may be effective in the treatment of PTSD.

This hypothesis was tested using a rodent model of PTSD in which rats were given inescapable foot shock in the presence of an unfamiliar object [71]. In this animal model, rats bury a stress-associated object when re-exposed to it several days later, a behavioral analogue of PTSD in humans. High frequency DBS of the basolateral nucleus of the amygdala (BLA) resulted in a decrease in burying behavior. In addition, the beneficial effects of DBS were observed even when it was performed after the establishment of the PTSD-like behavior [72].

Recently, resting state fMRI was used to show that activation of the amygdala in PTSD patients correlates with symptom severity [73]. Resting-state fMRI in PTSD patients also revealed increased functional connectivity between the amygdala, insula, and hypothalamus and decreased connectivity with the anterior cingulate gyrus and vmPFC [74–76]. It has been suggested that the vmPFC normally acts to inhibit the amygdala and that disinhibition results in overactivity of the amygdala leading to the symptoms of PTSD [77].

Human studies of DBS for PTSD are limited. In one case report, bilateral DBS of the BLA was performed for treatment of self-injurious behavior (SIB) in a 13-year-old autistic boy. DBS in this patient resulted in significant reduction in SIB which was sustained on follow-up [78]. In addition, DBS of the amygdala was not associated with any significant side effects such as seizures. A protocol was recently published to establish a pilot phase 1 randomized double-blind sham-controlled trial of bilateral DBS of the BLA to be performed in six combat veterans with PTSD [77]. Given the episodic nature of symptoms seen in PTSD and other anxiety disorders, novel stimulation techniques such as closed-loop DBS will play a greater role in the treatment of these disorders. Unlike present-day DBS which is applied in a continuous manner, closed-loop DBS would work on a contingency basis by detecting aberrant electrophysiological signals and preemptively responding with the appropriate amount of stimulation in order to prevent or abort a PTSD or anxiety attack [79,80].

Anorexia Nervosa

Anorexia nervosa (AN) is a psychiatric disorder characterized by a failure to maintain a minimum body weight , an intense fear of gaining weight and an abnormal perception of body image. Patients with AN often have comorbid depression, OCD,

and anxiety. Current treatments include a combination of pharmacotherapy and psychotherapy, although their efficacy has been shown to be limited. AN has the highest mortality rate among psychiatric disorders and there is no effective treatment [81]. Therefore, there is an urgent need to develop novel therapies for AN. Neuroimaging and preclinical data show that AN likely involves a disturbance in limbic networks common to mood and anxiety disorders. Thus, several of the limbic structures discussed previously have been targeted in the search for an effective neurosurgical treatment for AN.

The first lesion for AN was performed in 1950 using a prefrontal leucotomy one year after Egas Moniz was awarded the Noble Prize [82]. From 1950 to 1973, a total of 17 prefrontal leucotomies were reported for AN. With the advent of the stereotactic era, neurosurgical treatment of AN consisted of more focal lesions in the anterior cingulate cortex and the mediodorsal thalamus [83]. Recently, bilateral anterior capsulotomy was performed in a single patient with AN and comorbid OCD with an improvement in the patient's OCD symptoms and normalization of weight [84].

Preclinical animal studies have identified additional potential DBS targets. To date, there have been three animal studies on DBS for AN showing an increase in food intake by stimulating the ventromedial hypothalamus or medial NAc but no effect from stimulation of the lateral hypothalamus [85–87]. Given the complexity of the disease, these animal models, by necessity, only address food intake and do not directly address the affective component underlying AN.

A small number of clinical studies have investigated the use of DBS for AN. In one case report DBS of the SCC for depression resulted in an improvement in comorbid AN with normalization of the patient's weight after 2 years [88]. In another case report, bilateral DBS of the VS for OCD in a patient with comorbid AN resulted in an improved attitude toward food [89]. In addition to these case reports, there have been two case series to date. In a group of four adolescent patients, bilateral DBS of the NAc resulted in improvements in weight and resumption of school in three out of the four patients. Our group performed a trial of high frequency DBS of the bilateral SCC in six patients. At 6–9 months follow-up, the average weight was significantly higher than baseline in most patients, with significant improvements in depression and anxiety which enabled the patients to participate more effectively in psychotherapy [90]. In addition, PET imaging showed that DBS in these patients resulted in increased cerebral metabolism in the parietal lobe with decreased metabolism in the cingulate gyrus and bilateral insula [90].

The early and modest result from using DBS for AN should be interpreted with caution. AN has several particularly challenging and potentially confounding features including depression and OCD. It is a heterogeneous disorder with multiple subtypes and etiologies, and it is unclear for whom the procedure would be most beneficial. Furthermore, all studies to date have been open-label, and it remains unclear to what extent the observed findings are related to active versus long-term DBS. However, the metabolic changes observed as a result of DBS point to a long-term effect of DBS on limbic circuitry directly implicated in AN. Patient-specific neuroimaging combined with better animal models will be particularly important in identifying the most appropriate target for each patient, as well as which patients would benefit most from neuromodulation.

Table 26.1 Summary of brain areas targeted for DBS in human subjects for each psychiatric disorder

DBS targets for psychiatric disorders	
Disorder	Targets in human studies
Addiction	NAc, STN
Anorexia Nervosa	SCC, VC/VS, NAc
Depression	SCC, NAc, VC/VS, ALIC, LH, ITP, MFB
OCD	ALIC, VC/VS, NAc, ITP, STN
PTSD	Amygdala (proposed)

ITP inferior thalamic peduncle, *LH* lateral habenula, *MFB* medial forebrain bundle, *NAc* nucleus accumbens, *STN* subthalamic nucleus, *SCC* subgenual cingulate cortex, *VC/VS* ventral capsule/ventral striatum

Conclusions

It is now clear, from genetic, preclinical, and imaging data, that many psychiatric disorders are generated and maintained by abnormal network activity in the brain [91]. As for movement disorders, these conditions should also be amenable to treatment by methods that can restore or overcome their pathologic networks. At the present time, DBS offers the most direct method to access and modulate critical nodes within these dysfunctional limbic networks and the evidence summarized in this chapter suggests that DBS can be effective for many of these psychiatric disorders (Table 26.1). One major challenge of particular relevance to psychiatric disorders is the present difficulty in characterizing which patients are most likely to benefit from intervention. Often, the "failure" of treatment is due to our lack of understanding of the variation in the disease, the inability to identify the appropriate target for a given patient, or the lack of clinical tests to discern subtle treatment effects. Thus, advances in animal models, structural and functional neuroimaging, and advances in bioengineering will continue to improve our ability to better characterize the pathologic networks involved and to improve DBS technology to target these networks in the most efficacious and ethical manner.

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Chapter 27
Novel Interventions for Stroke: Nervous System Cooling

Patrick D. Lyden, Jessica Lamb, and Padmesh S. Rajput

Abstract Stroke accounts for more disability than any other neurological disorder and globally ranks as a leading cause of death. Neuroprotection must be developed, because although recanalization therapy benefits over half of all treated stroke victims, there remains substantial residual morbidity. A significant development in translational stroke research was the creation of the quantal bioassay for drug screening. Using the bioassay and other models, many have shown the benefit of therapeutic hypothermia for acute ischemia stroke in a variety of animal models, making hypothermia the most potent putative stroke therapy ever developed. Ongoing clinical trials will validate these promising translational findings.

Keywords Stroke • Therapy • Therapeutic hypothermia • Animal models • Ischemia • Hemorrhage • Guidelines

Introduction

Stroke accounts for more disability than any other neurological disorder and globally ranks as first, second, or third leading cause of death depending on the continent [1, 2]. Most importantly of effective stroke treatment, therefore, outranks nearly all other disorders but not cancer and heart disease. Neuroprotection must be developed, because although recanalization therapy benefits over half of all the treated stroke victims [3], there remains substantial residual morbidity. As new treatments for human stroke continue to fail in clinical trials [4], a growing chorus of critics challenges the validity of preclinical stroke models. Several obvious reasons for the translational failures in stroke should be considered before one rejects the validity of all models. For example, in the lab, test animals often receive putative stroke treatments

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immediately after ischemia onset—an impossible clinical scenario [5]. Patients are not anesthetized during stroke as the animals are, and most anesthetics have a variable cytoprotective effect; it would be reasonable to consider testing drug infusions only in awake animals [6]. Young adult male animals free of comorbidities such as hypertension and diabetes are preferred by most investigators so that outcomes are more reproducible and sample sizes can be minimized, but of course patients are older, of both genders, and have other disorders. Given these limitations of animal models, critics look at the vast number of failed human stroke trials and conclude that preclinical animal stroke modeling is useless [7].

On the other hand, similar and devastating indictments could be leveled against the clinical trials themselves [5]. The vast majority of human clinical stroke trials were designed for the convenience of the study sponsor and not based on the known science of the test drug [8]. For example, while the therapeutic window for a treatment may be only an hour to two following ischemia in test animals, the human trial may allow a 6 or even 12-h window from stroke onset to treat initiation [9, 10]. In many cases, the therapeutic concentration of the test drug that yields benefit in animals cannot be used in humans due to side effects; failure of 1/10 the known therapeutic dose should not surprise anyone, and there is no need to conclude that animal models fail to predict clinical outcomes [11]. Quite the contrary, such failures do in fact validate the use of preclinical stroke models because failure was predicted [8].

There is one critical success in stroke preclinical modeling and it deserves discussion. In 1985, Zivin and colleagues demonstrated for the first time that intravenous thrombolytic therapy with the recombinant human tissue plasminogen activator (rt-PA) ameliorated the behavioral effects of acute cerebral ischemia [12]. To accomplish this landmark study, the investigator created a new stroke model using autologous blood clot that was injected into the internal carotid artery (ICA). Previously, many labs had attempted to model stroke with a single injected blood clot embolus [13, 14]. The destination of the injected embolus, however, could not be predicted; proximal or distal cerebral artery or not even in the cerebrum at all. Also, the resulting infarction volume was highly variable. To overcome the unpredictability of the standard embolic clot model, Zivin had the insight to inject hundreds of microclots, all small enough to reach end-arterioles. While the location of any one microclot could not be predicted, the result of embolizing hundreds of microclots was highly reproducible. Further, rather than measuring the final infarction volume, the investigators measured the behavioral response of the animals (in this case rabbits) to the ischemia. To quantify the behavioral measurement, Zivin and Waud adapted the quantal bioassay to stroke [15]. It can be predicted that a small number of microclots will render *none* of the animals "abnormal" using a blinded assessment 48 h after embolization. A very large number of microclots will render *all* the animals abnormal. By using a graded range of microclot quantities, the investigator can generate a "dose–response" curve for the behavioral response to graded doses of injected microclots; to this data one can fit the logistic function (the logit test works just as well). The logistic function will generate two parameters for the fitted curve: the ED_{50} and the slope of the curve (Fig. 27.1). The ED_{50} is the "effective dose" of microclots that causes 50 % of the animals to score abnormal. From the slope the ED_{50}s of two treatments, the investigator can estimate the variance and compute a standard *t*-test.

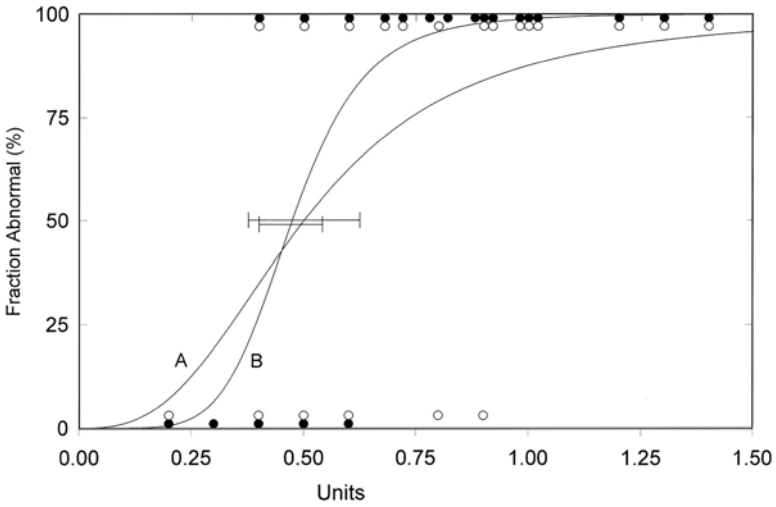

Fig. 27.1 The quantal bioassay. The bioassay uses a behavioral rating score for each animal and a graded insult, here labeled "units." Each animal is rated normal or abnormal; the *open circles* are from animals in Group A, and the *filled circles* Group B. The logistic function is fit by iteration to the scores [15]. The number of "units" that render 50 % of each group abnormal is the ED_{50}; the variance is derived from the slope of the fit curve. Two ED_{50}s are compared using a *t*-test

After embolization with microclots, thrombolytic therapy caused a significant increase in the ED_{50}, meaning the animals tolerated a much larger injection volume of microclots compared to placebo treatment. This landmark paper, along with others, led to the definitive trial of rt-PA for humans, published 10 years after Zivin's paper [3].

The quantal bioassay has been adapted into a wide variety of stroke models for the study of different central nervous system disorders. The bioassay allowed demonstration of neuroprotection after intracerebral hematoma [16, 17]. Central nervous system ischemia can be induced with aortic occlusion (causing spinal cord ischemia) for testing of neuroprotectants [18]. Another model that is more clinically faithful involves occlusion of the middle cerebral artery (MCA) for varying durations to generate the graded insults [16]. To illustrate, Fig. 27.2 shows bioassay curves derived from animals given a range of occlusion times and treated with whole body hypothermia, compared to normothermia or hyperthermia. A powerful and impressive effect is easily detected with only 22–25 subjects per group.

An essential advantage of the bioassay is that it utilizes behavioral outcome and is therefore quite clinically relevant because human stroke studies depend on behavioral outcome measures (e.g., The NIH Stroke Scale or the modified Rankin Score), not infarction volume. Another advantage is the use of graded insults in the bioassay; in standard preclinical stroke models a single, usually mild, dose of injury is applied so as to allow greater survival of the animals. This mild injury does not replicate the human situation in which different patients suffer different—mild, moderate or severe—strokes. The bioassay involves a range of severities and thus allows the investigator to study the putative therapy over the range of expected insults.

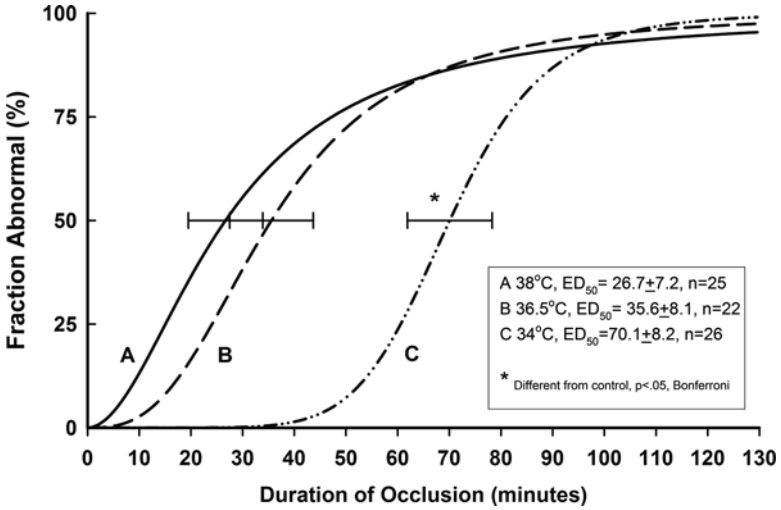

Fig. 27.2 Neuroprotection with hypothermia. A quantal bioassay was used to demonstrate the effect of whole body hypothermia on anesthetized rats undergoing graded durations of tMCAo. As in Fig. 27.1, the ED_{50} and variance were estimated for each group. Animals cooled to 34 °C temporalis muscle temperature showed a significantly increased ED_{50}, i.e., they tolerated ischemia of much longer durations. Warming the animals slightly decreased the ED_{50} but this was not statistically significant [16] (Used with permission; Elsevier. Experimental Neurology 1997;147:346–352)

A final lesson from the quantal bioassay derives from knowing that very few laboratories have adopted the method. The method is sound and faithfully reproduces the disease under study but the typical clinician scientist has a hard time conceptualizing a model that involves hundreds of simultaneous microemboli, a rare clinical scenario, or a graded range of insults with a nonlinear curve fit to the data. Thus, in the vast majority of labs the preference has been to model human stroke with a single vascular occlusion, despite all the disadvantages noted above. The success of the bioassay suggests that the choice of the animal model used for any given experiment should be dictated by the clinical question. In 1985, Zivin's question was "Does intravenous thrombolysis promotes behavioral recovery from cerebral ischemia" and the model elegantly answered that question [12]. Another question, such as "Does intravenous thrombolysis causes cerebral hemorrhage after embolic stroke" required a different model [19]. Another question, "Does neuroprotection reduces the number and size of small infarctions after intra-arterial instrumentation" dictated yet another model, in which Tymianksi et al. created a model that could be performed nearly identical in primates and in humans [20]. If we are to move the field of preclinical stroke research forward significantly, we must move away from models that reproduce the human situation with high fidelity and toward models that better answer the specific question at hand. To decide whether any putative therapy will work in humans depends on well-designed human trials and not on the results of the preclinical studies. Well-designed preclinical

studies can answer fundamental questions related to the biology of the disease, the effect of treatment on key outcomes, and the nature of obvious side effects of the treatment. The field, however, remains focused on more conventional models.

Optimized Stroke Modeling Using Conventional Models

Conventional methods of drug screening in an animal model of stroke are often conducted by occluding the MCA (MCAo) [21] and delivering the test substance by intravenous or intraperitoneal injection. The MCA can be occluded permanently, pMCAo, or temporarily, tMCAo. Most stroke patients suffer permanent vascular occlusion and the investigator seeking to model this could use the pMCAo animal approach. On the other hand, some patients are treated with recanalization therapy and suffer injury related to reperfusion [22, 23]. An investigator seeking to model this biology would use a tMCAo approach. The oldest stroke model to gain wide acceptance involved pMCAo using clips applied to the MCA via the orbit [24]. A clip that is released allows tMCAo but a better approach uses a nylon catheter inserted via the ICA [25]. Measurements should include behavioral outcomes as well as traditional infarct volumetry [26].

These methods allow high-throughput modeling, as they are easy to perform, but the distribution of the test substances includes the entire body and can be influenced by peripheral drug metabolism [27]. A large quantity of the injected test substance could be metabolized or excreted by the lungs, liver, or kidneys before reaching the brain mandating a much larger dose of test substance to achieve an effective level in the brain. Such larger doses might be accompanied by side effects and complications in other tissues or simply might be to too expensive. To solve these issues, we proposed a method for implanting a catheter in the carotid artery that will allow deliver of smaller quantities of test substances directly to ischemic brain [28].

Optimized Stroke Modeling with the Rodent Filament Model

Adult male Sprague Dawley rats weighing 290–310 g are typically used because of their detailed vascular and neuroanatomy, their ease of handling, and a well-documented repertoire of measurable behaviors. Fisher, Long Evans, Kyoto, and Wistar rats have been used with great success as well. In the future, all studies should include males and females, even though females may respond to MCAo variably [29]. Despite the increased costs, at least some studies of a new drug must include aged animals [30]. Animals should be housed under standard conditions (21–23 °C, 12 h light–dark cycle) with unlimited access to standard food and water. Variability in the resulting infarct volume can be greatly reduced if the nylon filament used to occlude the artery is standardized [28]. A 4-0 Ethilon monofilament suture can be heat blunted by waving each filament briefly through a flame; the tip diameter should be measured under a microscope (Fig. 27.3) and recorded for later use.

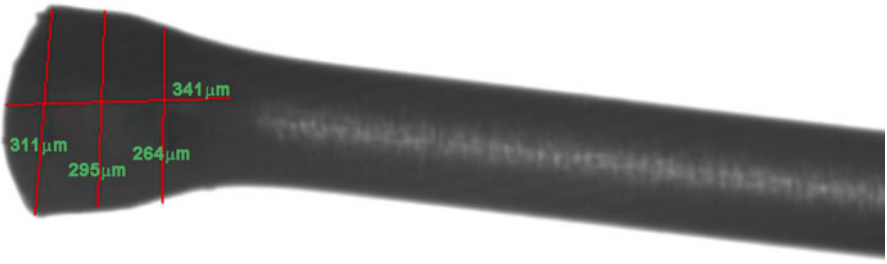

Fig. 27.3 Heat blunted nylon filament. Most stroke labs use heat blunted nylon filaments to promote better entry of the filament into the intracranial circulation without puncturing the arterial wall. Each filament is measured three times under the microscope, and the largest diameter obtained is recorded

We typically prepare doses of filaments at one time and store them for use over the ensuing months. To increase the likelihood of successful MCA occlusion (a known issue with this method), a heat blunted filament should be selected based on the previously measured tip diameter, ranging between 290 and 310 μm. A 1:1 ratio correctly selects an ideal suture size, i.e., a 300 μm suture tip will properly occlude the MCA in a 300 g rat. The suture is marked 1.7 cm from tip using a wax pencil to guide placement depth into the ICA. Anesthesia should be induced with 4 % isoflurane, in 70 % N_2O and 30 % O_2, and maintained at 2–2.5 % after placing the animal securely in a nose cone. We strongly recommend—although some disagree—intraperitoneal (IP) injection of 0.05 mg/kg atropine and 0.4 mg/kg carprofen subcutaneously (SQ); the eyes should be lubricated with a petrolatum ophthalmic ointment. A servo-controlled warming blanket is needed to maintain core body temperature to 37 °C based on rectal or temporalis temperature [31]. The fur on the ventral cervical area from the mandibles to the sternum is shaved and another area dorsally between the scapulae also is shaved (catheter exit site). From both areas, loose hair can be removed with a clothing lint roller, and the skin swabbed with betadine, followed by 70 % alcohol. All materials and the surgical procedures should be performed under aseptic conditions.

Although the rodent is large enough to do this surgery unaided, we prefer to use the operating microscope with 7× magnification to guarantee proper catheter placement and minimize damage to adjacent structures such as the vagus, glossopharyngeal, and hypoglossal nerves [32]. Via a midline skin incision and blunt dissection, a retractor is positioned so that the sternomastoid muscle and mandibular glands are gently retracted laterally. Various branches are ligated with 4-0 silk and loops of 4-0 silk are placed around the ICA and ECA but not tightened. The CCA is occluded and the selected monofilament is inserted into the ECA pointing toward the CCA and then curved up toward and into the ICA. The filament is advanced until the mark (1.7 cm) reaches the bifurcation of the ICA and ECA. The occlusion time is noted and duration of occlusion is set from this start time.

Through the same operative view, a catheter can be placed in the CCA for later intra-arterial infusions. The catheter is introduced through the existing incision in the ECA and advanced retrograde into the CCA as far as possible. Using loops already placed to secure the ECA for the filament insertion, the catheter is secured

carefully so as not to cause CCA occlusion. Via a ventral exit wound, the catheter is pulled through a subcutaneous tunnel and secured. The infusion catheter can be used before, during, or after MCAo, while closing all the skin incisions. To deliver jugular infusions, catheters can be placed through a small venotomy. A silicone catheter is advanced approximately 12 mm and secured with two sutures; one distal to the venotomy and one proximal. The catheter can be tunneled around the side of the neck and secured as described for the arterial catheter.

For all versions of the MCAo model, the animal should be transferred to a pre-warmed recovery chamber after anesthesia. At the end of a prespecified duration of occlusion the necessary neurologic exams are preformed (forelimb withdrawal, twisting to one side when suspended by tail, and circling) and then anesthesia is induced as previously described and the ventral skin incision is reopened. A 4-0 silk suture is placed around the ECA/catheter and gradually tightened as the nylon occluding filament is removed slowly from the ICA. Although we attempted for many years to develop a system in which the filament can be removed from the animal while awake, this has never worked, and it seems that two anesthetic events are unavoidable.

We modified a standardized examination of rat neurologic function from a published method [33]. The animal is examined during the MCAo (if the planned duration is long enough), again after reperfusion (removal of the nylon filament), and at 24 and 48 h. First we look for the animal to withdraw a forelimb when suspended by its tail. While suspended, we also look for the animal to twist toward the contralateral side of the occlusion. Finally, we observe the animal for circling behavior. For each abnormal finding, we give the score of 1 point for a total possible score of 3. After a predetermined reperfusion period, the animals are sacrificed with an overdose of anesthetic administered intraperitoneally, followed by trans-cardiac perfusion with warm 0.9 % saline. The brain can be removed in a standard manner and prepared for sectioning, or for protein or nucleic acid extraction.

Optimized Stroke Modeling Advantages

Our combined occlusion/infusion model takes advantage of the fact that a nylon occluding monofilament leaves a residual flow of about 20 % in the hemisphere so there is sufficient flow to carry an infused substance from the carotid artery to the branches of MCA [34]. We have shown that this intra-arterial infusion method delivers considerably more study drug into the ipsilateral, ischemic hemisphere than the intravenous infusion [28]. When we delay drug infusion until after de-occlusion, all of the restored blood flow carries the test substance anterograde into the ischemic territory.

The intra-arterial delivery approach enables the investigator to target the ischemic region through an endogenous mechanism of blood brain barrier opening and increases the efficiency of drug delivery—as a result the total amount of substance needed is reduced [35]. Such delivery efficiency is important when test substances may be expensive or in short supply. Further, smaller test infusions may reduce

potential systemic side effects. Finally, intra-arterial delivery allows the investigator to infuse the substances in a controlled manner at a given time for a specified duration without confounds from variable absorption (e.g., after intraperitoneal administration) or variable metabolism (e.g, after intravenous administration). We have demonstrated the utility of carotid artery infusions in several publications; in fact, some of the experiments would have been impossible without the use of the carotid artery infusion catheter due to the peripheral side effects of the test drugs [36, 37].

Therapeutic Hypothermia

Therapeutic hypothermia is the most potent neuroprotective therapy ever studied in experimental cerebral ischemia. Cooling the brain as little as one degree Celsius significantly alters brain responses to ischemia [31]. Therapeutic hypothermia exerts multiple effects at multiple stages of the ischemic cascade, many of which involve temperature dependent mechanisms (Fig. 27.4) [38]. Today, in all experimental cerebral ischemia studies, brain temperature must be rigidly controlled to avoid confounding effects [31]. Over the past decade or two, single mechanism and single target stroke therapies have failed to improve outcome in patients, repeatedly

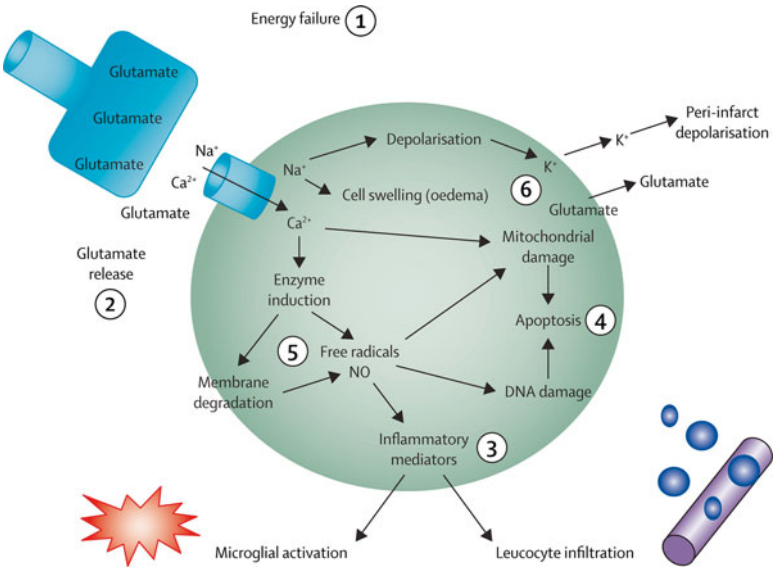

Fig. 27.4 Mechanisms of neuroprotection during hypothermia. Therapeutic hypothermia provides neuroprotection after cerebral ischemia via several mechanisms. (1) Decreasing cerebral metabolism but preserved cerebral blood flow. (2) Suppressing glutamate release from presynaptic excitatory neurotransmitters. (3) Reducing inflammatory responses from leucocytes, peripheral monocytes, and microglia. (4) Disrupting apoptosis. (5) Reducing free radical production and release. (6) Reducing cerebral edema (Used with permission [38] Elsevier. Lancet Neurology 2013;12:275–284)

[39]. In addition to reducing temperature dependent processes such as proteases, caspases, endonucleases, lipases, and metalloproteinases, hypothermia inhibits inflammatory responses such as leukocyte migration/lymphocyte activation and minimizes free radical generation [40]. Brain metabolism—consumption of oxygen and glucose—dramatically drops under hypothermic conditions, thus conserving resources and prolonging penumbral survival but regional cerebral blood flow is preserved [38, 40–45].

No other neuroprotectant has been studied in such a broad and deep range of animal cerebral ischemia models as has hypothermia. In a large survey of preclinical work, an independent group rated the rigor and quality of preclinical hypothermia studies as excellent [44]. Of all putative neuroprotectants studied, hypothermia ranks among the "best," meaning there are sufficient numbers of high quality studies to suggest eventual clinical success. A number of preclinical studies meet the RIGOR guidelines [39]. There is a clear and consistent benefit of hypothermia seen prominently in higher quality papers that included randomization, blinding, and both histological and functional outcomes [44, 46].

Translating this potent protective effect to clinical applications has proven problematic. Multiple studies have documented powerful protection with therapeutic hypothermia after accidental neonatal hypoxic-ischemic injury [47, 48]. Early studies of global cerebral ischemia after cardiac arrest confirmed powerful protection after therapeutic hypothermia [49, 50]. National and International guidelines recommend therapeutic hypothermia for selected survivors of cardiac arrest, with profound benefits seen anecdotally. More recently, however, a study comparing target temperature of 33–36 °C failed to demonstrate significant effects in cardiac arrest patients [51]. This recent trial differed from earlier trials with respect to cooling duration and time to reach target depth, in that target was not reached until much later than in the prior trials. Nevertheless, this single study profoundly influenced behavior in Europe and to some extent in the US; several major programs have eliminated therapeutic hypothermia programs.

On the other hand, hypothermia studies in patients with traumatic brain injury failed to show benefit [52], despite a clear and proven effect on edema and intracranial pressure [53]. Therapeutic hypothermia trials in acute myocardial infarction patients also failed, although reanalysis suggests a protective effect if hypothermia begins prior to recanalization [54–56]. Although negative on a primary endpoint, the CHILL-MI trial demonstrated a significant benefit in patients with anterior wall ST segment elevation myocardial infarction who were recanalized after achieving target temperature [57].

Clinical trials of therapeutic hypothermia for acute stroke are ongoing and were designed based on the available preclinical literature. I select a clinical trial design; systematic review of the preclinical literature suggests a few key points [58]. First, *therapeutic hypothermia succeeds when combined with recanalization/reperfusion*; beneficial effect after permanent ischemia is seen but with smaller effect size [38, 59–63]. This observation suggests that the optimal clinical design should combine hypothermia with recanalization therapy, such as IV rt-PA or intra-arterial thrombectomy [58]. Second, *therapeutic hypothermia usually shows greater benefit the earlier it begins*. Therapeutic hypothermia started before or soon after stroke is most

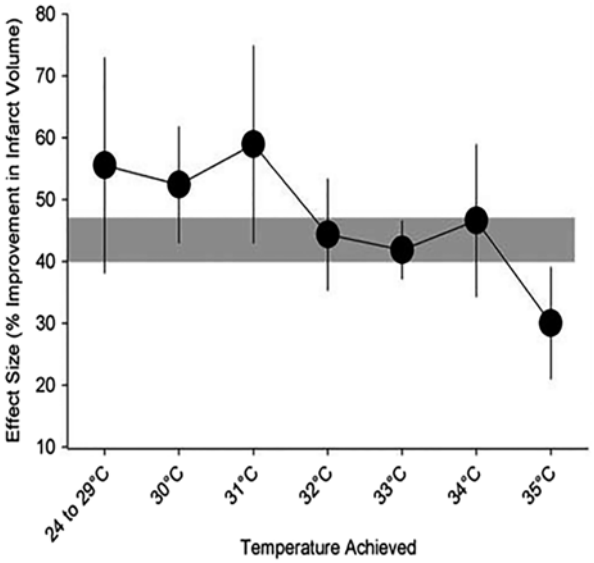

Fig. 27.5 Meta-analysis of animal studies of hypothermia for stroke. Over a broad range of target temperatures, effect size was estimated from the results in 3353 animals. Infarct volume was estimated in different ways, but the effect size was standardized as the improvement as a percent of the untreated volume. The *gray band* demarcates the 95 % confidence interval for the overall estimate of efficacy and the *vertical error bars* represent 95 % confidence intervals at each point. There is a notable trend toward greater benefit at lower target temperatures (Used with permission [44], Nature Publishing. J Cereb Blood Flow Metab 2010; 30:1079–1093)

effective, but even when started as late as 3 h after stroke, therapeutic hypothermia remains considerably protective [61, 64]. Thus, the time window for beginning hypothermia should be short, so that treatment starts very soon after recanalization treatment. Third, depth of cooling appears to influence outcome significantly, with *33 °C seeming to provide more benefit than 35 °C* as illustrated in Fig. 27.5; there is controversy on this point, however, and clinical studies are needed for an assessment of the effect of cooling depth on outcome [51, 60, 62, 65–69]. Fourth, duration of cooling affects benefit: *longer durations provide greater benefit* [61–63, 70, 71]. In particular, it appears that longer cooling durations are especially important if cooling onset is delayed [64]. Given the enormous numbers of patients suffering acute global or focal ischemia, and given the great potential benefit of therapeutic hypothermia, there is a compelling and urgent need to optimize the key parameters of therapeutic hypothermia: *target-depth* temperature, *duration*, and maximal *delay* after which treatment is futile.

Progress in experimental therapeutic hypothermia has been hampered by the absence of simple, rapid, and inexpensive models [44]. The best animal model, exemplified by a series of studies from Corbett and Colbourne, requires a complex set-up involving implanted telemetered thermistors, radio-controlled water misters and cage-mounted servo-controlled fans [64, 70]. The data and science are elegant, but the setup does not allow extensive modeling for translational research and implementation.

Important findings from the Corbett/Colbourne lab so far are that (1) deeper hypothermia to 33 °C is probably better than 35 °C and (2) if the onset to therapeutic hypothermia is delayed, the duration must be longer to obtain the same effect [63]. Other labs have confirmed this interesting relationship between delay and needed duration [72, 73]. The mechanism of the delay–duration effect is not known, and there is a compelling need for detailed studies of the differential effects of target depth, delay, and duration on these different elements of the neurovascular unit (NVU).

To separately study the interactions between ischemia and treatment, we created a novel in vitro model to study elements of the NVU. Up until recently, studies of neuroprotection addressed the brain as a homogenous unit, with the tacit assumption that all elements responded similarly. The recent description of the NVU—comprising neurons, astrocytes, endothelial cells and pericytes—allows more focused investigation [74]. For example, in our previous in vivo work, we have shown differential effects of various neuroprotectants on endothelial cells, astroctyes, and neurons [37, 75]. Seeking to explore these effects in vitro, we now have cultured three cell types. In a proof-of-concept experiment, we found that cultured neurons are significantly more vulnerable to oxygen–glucose deprivation (OGD) than other elements of the NVU. We cultured neurons, astrocytes, or endothelial cells from rats and applied standard OGD of varying durations. From literature concerning selective vulnerability, we predicted neurons would be most vulnerable, followed by astrocytes and endothelial cells, $N<A<E$. Surprisingly, as shown in Fig. 27.6, we found $N<E<A$. In fact, astrocytes showed 80 % cell death after 10 h of OGD, compared to 2 h for neurons and 6 h for endothelial cells.

We sought to test various conditions like *duration*, *delay*, and *depth* of hypothermia in each cellular element of the NVU. Primary neuronal and endothelial cells were isolated from E16 to E17 embryos and survived for 8–10 days for further

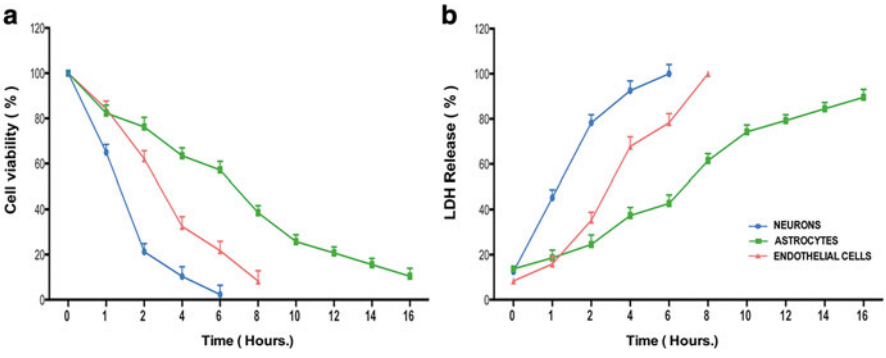

Fig. 27.6 Elements of the neurovascular unit respond differently to oxygen glucose deprivation. In relatively pure culture, each element of the NVU—neurons, astrocytes, and endothelial cells—was subjected to increasing durations of OGD. Cell death was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays [76, 77]. In Panel (**a**), cell viability (MTT assay) is shown for varying durations of OGD on the *x*-axis. In Panel (**b**), cell death is represented by LDH release for varying OGD durations. Each time point is shown as the mean ± SE for at least six culture plate wells. Neurons exhibited the greatest vulnerability, with over 80 % cell death by 2 h compared to 6 h for endothelial cells and over 10 h for astrocytes

experiments. We used OGD injury to test for cytoprotective effect of hypothermia. We measured the protective effect of hypothermia (33 or 35 °C) after 2 h of OGD in primary neuronal cultures for various durations (2, 6, or 24 h). As shown in Fig. 27.7 Panel a, immediate hypothermia (no delay) conferred neuroprotection at

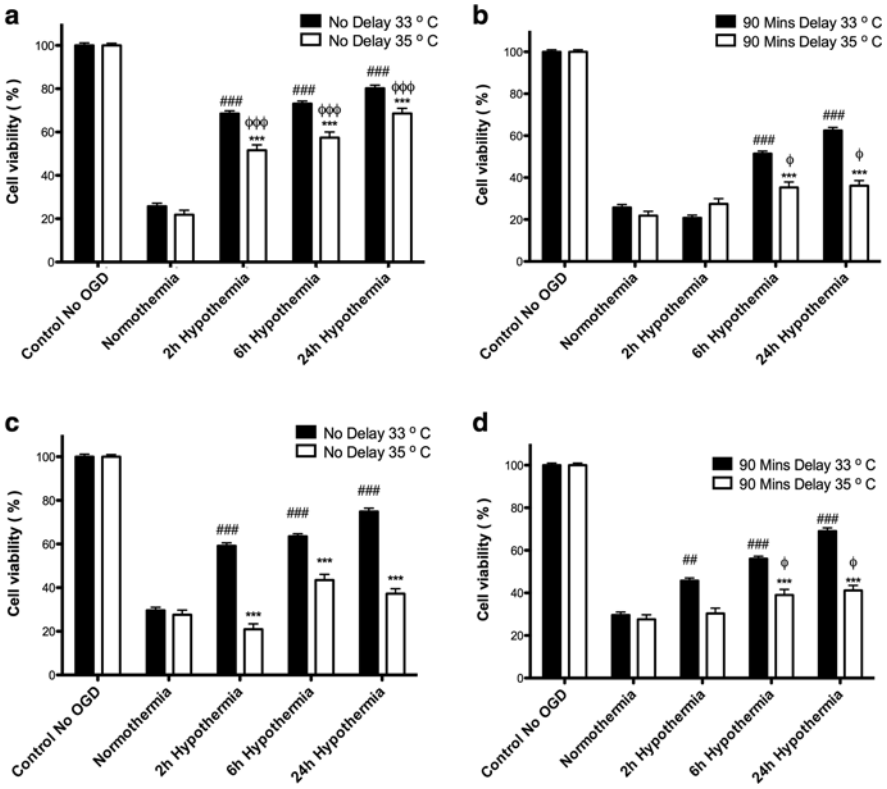

Fig. 27.7 Effect of hypothermia on neurons and astrocytes during oxygen glucose deprivation. Primary neuronal cells and astrocytes were cultured and then subjected to OGD; wells were randomly assigned to normothermia or hypothermia. We measured the protective effect of hypothermia (33 or 35 °C) after 2 h of OGD in primary neuronal cultures and after 10 h of OGD in astrocytes. We studied various durations (2, 6, or 24 h) of hypothermia started either immediately or 90 min after reversal of OGD. As shown in Panel (**a**), immediate hypothermia (no delay) conferred neuroprotection on neurons at all treatment durations (### $p<0.001$, 33 °C vs. normothermia; ϕϕϕ $p<0.001$, 35 °C vs. normothermia). Target depth 33 °C was superior to 35 °C at all treatment durations (*** $p<0.001$, 33 °C vs. 35 °C). As shown in Panel (**b**), when hypothermia was initiated 90 min after 2 h OGD treatment for only 2 h there was no benefit, but hypothermia for 6 or 24 h duration showed significant protection of neurons (### $p<0.001$, 33 °C vs. normothermia; ϕ $p<0.05$, 35 °C vs. normothermia) and again 33 °C hypothermia provided significant neuroprotection when compared to 35 °C hypothermia (*** $p<0.001$, 33 °C vs. 35 °C). In Panels (**c**) and (**d**), the result of hypothermia started after OGD in astrocytes reveals very similar findings. Using the MTT cell viability assay, hypothermia at 33 °C protected astrocytes after no delay regardless of duration (### $p<0.001$, 33 °C vs. normothermia), whereas 35 °C was not protective. At all durations, 33 °C was superior to 35 °C

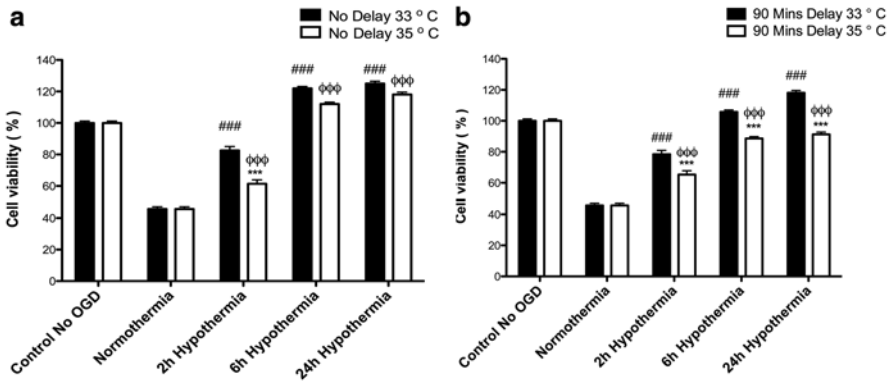

Fig. 27.8 Effect of hypothermia on endothelial cells after oxygen glucose deprivation. Similar to Fig. 27.7, we cultured endothelial cells subjected to 4 h OGD and then cells were treated with either 33 or 35 °C hypothermia for 2, 6, or 24 h beginning immediately (Panel **a**) or 90 min (Panel **b**) after reversal of the OGD. Cell viability was measured using MTT assay. Both 33 and 35 °C treated cultures showed significant cell survival at no delay, compared to normothermia (### $p<0.001$, 33 °C vs. normothermia; ϕϕϕ $p<0.001$, 35 °C vs. normothermia). However, 33 °C was better at 2 h when compared to 35 °C (*** $p<0.001$, 33 vs. 35 °C). For cells treated with hypothermia after 90 min delay (Panel **b**) both 33 and 35 °C showed significant cell survival compared to normothermia (### $p<0.001$, 33 °C vs. normothermia; ϕϕϕ $p<0.001$, 35 °C vs. normothermia). In the 90 min delay experiment (Panel **b**), hypothermia at 33 °C was superior when compared to 35 °C at all tested durations treatment (*** $p<0.001$, 33 vs. 35 °C). In all experiments, the results were analyzed using two-way ANOVA and the Dunnett's test for post-hoc comparisons

all durations, but in Panel b, when hypothermia was initiated 90 min after 2 h of OGD for various durations, 6 and 24 h but not 2 h durations showed protection. Deeper target temperature of 33 °C hypothermia provided significant neuroprotection when compared to 35 °C hypothermia. Endothelial cells were subjected to 4 h of OGD followed by hypothermia for 2, 6 and 24 h with no delay or 90 min delay. As shown in Fig. 27.8, we see similar protection by hypothermia in endothelial cells after OGD for various durations. These data confirm in vitro the prior suggestion from in vivo observations that a delay can be overcome with deeper hypothermia for longer duration [63, 72, 73]. This novel data provide critical insight—if replicated—that will immediately and clearly inform ongoing clinical trials of therapeutic hypothermia for both cardiac arrest and stroke [58]. Also, confirmation in an in vivo model is necessary.

Fig. 27.7 (continued) (*** $p<0.001$, 33 °C vs. 35 °C). After 90 min delay (Panel **d**) 33 °C was effective at all durations (## $p<0.01$, ###, $p<0.001$, 33 °C compared to normothermia), whereas 35 °C was marginally effective at 6 and 24 h (ϕ $p<0.05$, 35 °C vs. normothermia.) After 6 or 24 h delay, 33 °C was superior to 35 °C (*** $p<0.001$, 33 °C vs. 35 °C). In all experiments, the results were analyzed using two-way ANOVA and the Dunnett's test for post-hoc comparisons

There are some technical barriers to animal models of therapeutic hypothermia, most importantly, the choice of temperature monitoring approach. Previously, we have shown close correlation (Fig. 27.9) between cortical brain temperature and temporalis, or core body temperature by comparing telemetered brain temperature to core body temperature [16]. Using the quantal bioassay method described above (Fig. 27.3), we demonstrated considerable neuroprotection from cooling to 34 °C temporalis (33 °C brain) for as short as 2 h [16, 78, 79].

We recently developed a perivascular approach to therapeutic hypothermia in rats by placing an intravascular cooling catheter adjacent to the inferior vena cava, illustrated in Fig. 27.10. The advantage of perivascular cooling animals is that the technique is simpler and less expensive than surface cooling. Perivascular cooling allows very precise control of core body temperature (Fig. 27.11). The endovascular approach to therapeutic hypothermia, while commonplace in clinical trials, has not been previously developed in small animals [80].

Short periods of hypothermia are easily induced with topical alcohol and a fan (evaporative cooling) or with cooling pads (convective cooling) [62, 72].

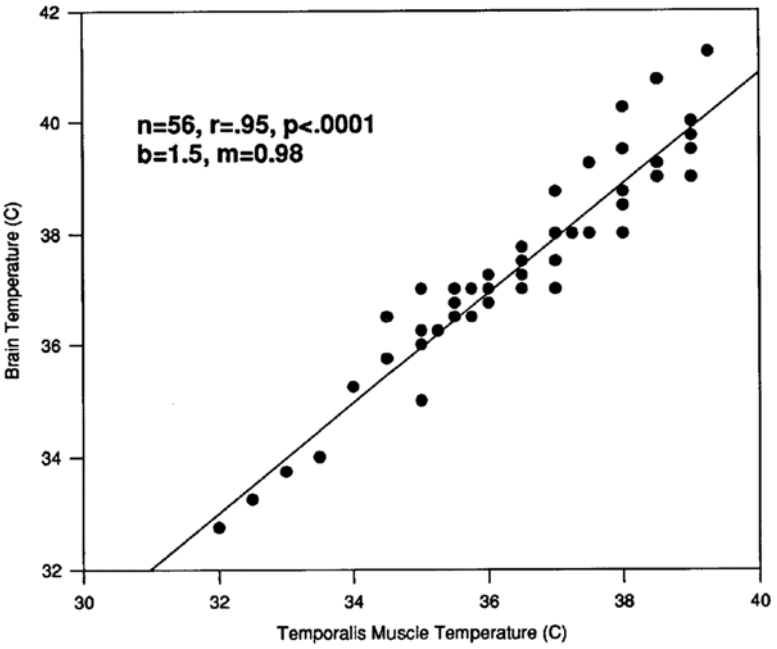

Fig. 27.9 Relationship between brain and temporalis muscle temperature. Right temporalis muscle temperature was monitored via a thermistor probe (Malinkrodt Anesthesia Products, Inc., St. Louis, MO) linked to a controller (YSI Model 73 ATD, Yellow Springs Instrument Co., Yellow Springs, OH) and a heat lamp to maintain brain temperature normothermic. In this study ($n=4$), temperature probes were implanted in the brain and muscle so that temporalis and brain temperature could be recorded simultaneously. There is a close correlation but the muscle temperature is about 1.5 °C lower than that of brain (Used with permission [16]; Elsevier. Experimental Neurology 1997;147:346–352)

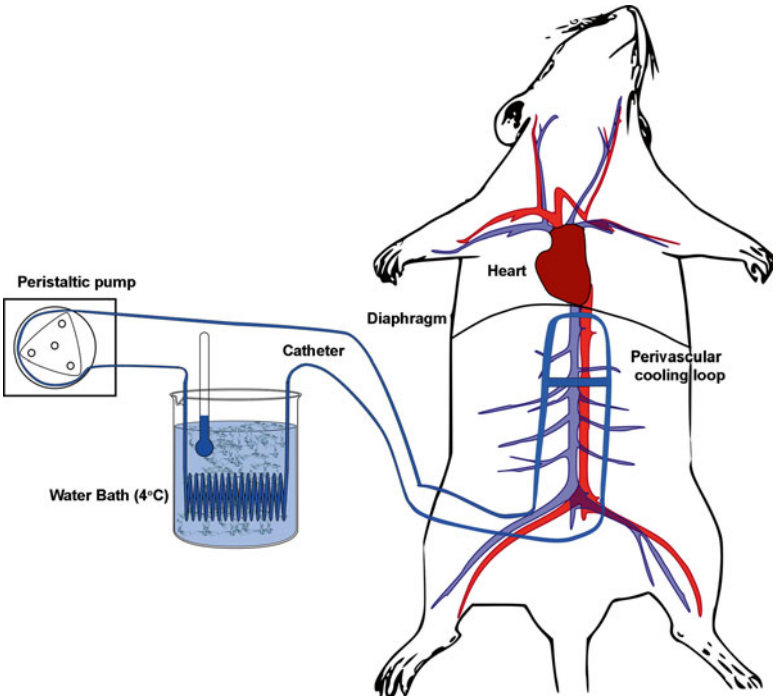

Fig. 27.10 Experimental setup for perivascular cooling a rat. A silicone catheter is secured in place around the inferior vena cava. The tubing exits the animal dorsally and is connected to a peristaltic pump. Extra tubing is coiled and placed into an ice water bath. Temperature can be measured from a probe placed in the temporalis muscle or rectum. By altering the flow rate on the pump, the body temperature can be precisely controlled manually

Prolonged cooling (up to 48 h) is possible only with thermistor-controlled water misters and fans. Surface cooling induces a stress response in the animals and control around the target-depth temperature is difficult. Recent studies have also shown effective hypothermia and neuroprotection by using TRPV-1 agonist (rinvanil) or an agonist active against the neurotensin receptor 1 [81, 82]. However, repetitive use of drugs might result in receptor desensitization or neurotoxic effects due to excessive doses and prolonged durations of hypothermia have not been studied to our knowledge. A simpler cooling approach potentially could open the research field to more labs that do not have the time or money to invest in the complex telemetry and cage system required for servo-controlled surface cooling with computer-controlled fans and misters. A simpler cooling model would allow more rapid translational studies of the optimal depth, delay, and duration for therapeutic hypothermia.

Clinical trials with hypothermia confirm that therapeutic hypothermia is well-tolerated, feasible, and safe. Endovascular cooling methods appear to provide for faster cooling compared to surface technology [83–85]. In unanesthetized patients, endovascular cooling is more tolerable than surface cooling because we apply skin

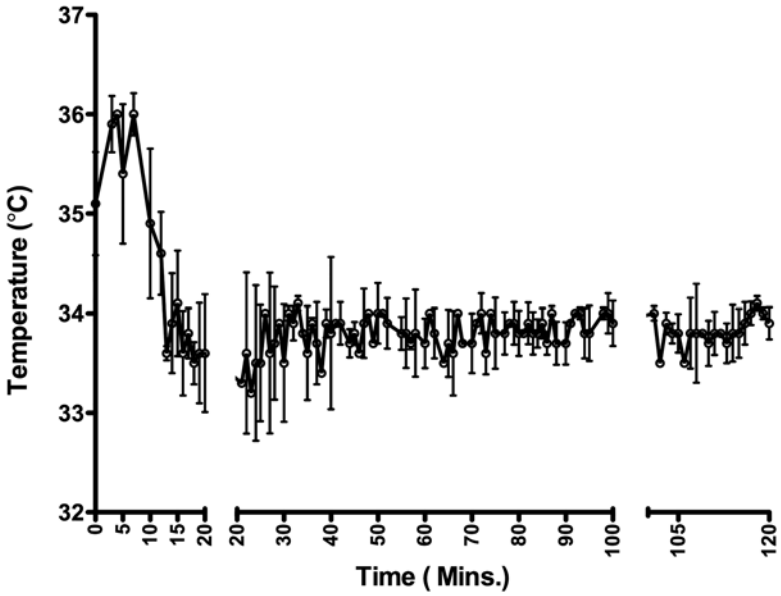

Fig. 27.11 Tight control of body temperature at 35 °C using perivascular cooling. Using the setup described in Fig. 27.10, we obtained body temperatures from a rectal probe in eight animals undergoing tMCAo using the filament occlusion model described earlier in this chapter. Each data point is the mean ± SE at one time point. Quite precise adjustment of the body temperature is achievable with this model

counter-warming for patient comfort [86]. Considerable previous experience [87–90] provided the foundation for several elements of the current stroke cooling protocol for acute ischemic stroke [58, 91–93].

Combination with Thrombolysis The rationale for using therapeutic hypothermia only in rt-PA-treated patients derives from preclinical data showing that therapeutic hypothermia is more effective after temporary MCAo compared to permanent MCAo [66, 94, 95]. In prior clinical trials, the rate of sICH was lower in the hypothermia groups, although no statistically significant benefit has yet been demonstrated [91]. There have been no reports of patients developing coagulopathy during therapeutic hypothermia to 33 °C or above. There have been no retroperitoneal hematomas and no significant groin puncture site hemorrhages from inserting an endovascular cooling catheter after intravenous thrombolytic therapy. Surveillance with lower extremity ultrasound showed the catheters do not induce deep venous thrombosis, although DVT is found in the paretic limbs of stroke patients [90, 91].

Determinants of Cooling Using multivariable analysis, we identified the patient features that determine cooling rate and adequacy [93]. Age and body surface area determine cooling rate: older patients cool more quickly and heavier patients cool more slowly. Cooling adequacy—measured as the time-to-target and depth of cooling—depends mostly on body surface area. Both cooling rate and adequacy depend on the degree of shivering control. Thus, the key to successful early cooling

is to begin endovascular cooling as soon as possible with an appropriately sized cooling device and to aggressively control shivering.

Permissive Hypothermia Based on experience gained in the previous trials, we developed the therapeutic concept of "permissive hypothermia.". We observed that patients tend to reach a plateau temperature within 2 h of cooling start and few patients achieve temperatures much lower. Aggressive upward titration of the meperidine infusion only raises the risk of aspiration, through respiratory suppression, without significantly lowering the target temperature reached. Thus, investigators at the bedside are asked to increase the meperidine infusion rate—if needed for shivering control and patient comfort—but not beyond the point of respiratory suppression. If shivering and discomfort continue, despite maximally tolerated meperidine, the clinician raises the target temperature from 33 to 35 °C, in half-degree increments. Therefore, we predict that the final ICTuS 3 treated group will include a range of final temperatures, centered on a median of 34 °C, as illustrated in Fig. 27.12 for the first 41 patients cooled in the ongoing ICTuS 2 trial.

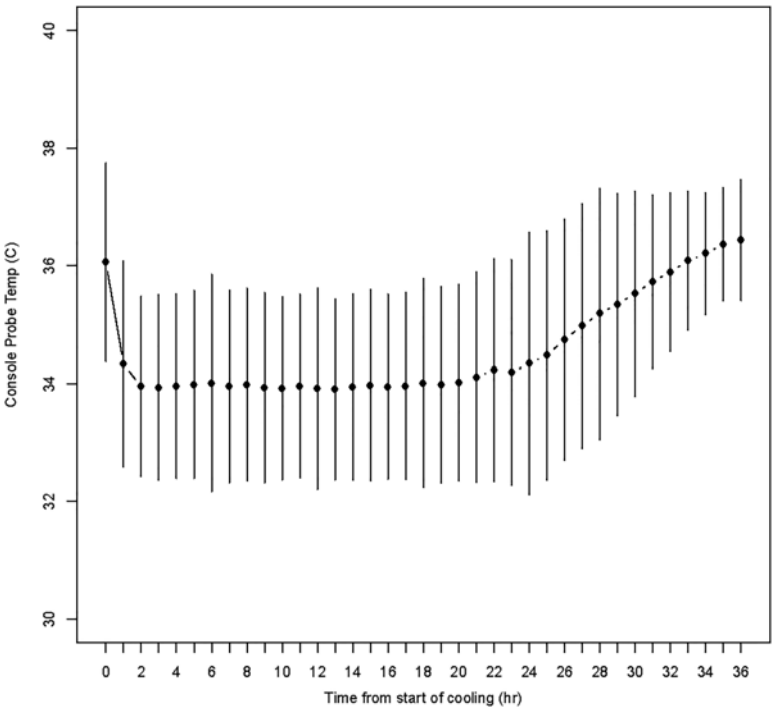

Fig. 27.12 Tight control of body temperature at 34 °C using endovascular cooling. In the ongoing ICTuS 2/3 trial (NCT01123161), patients are cooled with an endovascular catheter to a target temperature of 33 °C measured with a thermistor placed in the inferior vena cava. In this graph, we present the mean ± SE temperature for the first 41 patients treated in the trial. The typical temperature reached is around 34 °C, reflecting the fact that in this still ongoing blinded trial we cannot censor patients who fail to receive the treatment to which they were randomized. Nevertheless, the data indicate that most patients do reach a cooling plateau quickly

Conclusions

Translational neuroscience over the next decade promises to bring new technologies to patients with the most common disabling neurological conditions. Stem cells, exosomes, gene sequencing, therapeutic gene silencing, nanotechnology, high-field magnetic resonance imaging, functional neuroimaging, and a host of other technologies all promise to bring exciting possibilities to the patient's bedside. Yet, the oldest known neuroprotectant, therapeutic hypothermia still retains its position as the most powerful treatment available for the treatment of acute neurological injury. Therapeutic hypothermia has not yet entered the daily armamentarium, but current studies of therapeutic hypothermia for spinal cord injury, stroke, brain trauma, and intracerebral hemorrhage are underway. Not sexy, novel, or new, therapeutic hypothermia may hold the greatest promise for meaningful implementation sooner rather than later.

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Chapter 28
Rehabilitation Strategies for Restorative Approaches After Stroke and Neurotrauma

Bruce H. Dobkin

Abstract For acute, subacute, or chronic stroke, and neurotrauma, a range of rehabilitation strategies will be essential to optimize possible benefits of molecular, cellular, and novel pharmacological restorative approaches. The neurorehabilitation strategies must be chosen to engage the targeted networks of these novel approaches, drawing upon studies of motor and cognitive learning-related neural adaptations that accompany progressive practice. Regulatory agencies and the pharma/biotech industry will need to keep an open mind about the likely synergy that will come from interleaving repair strategies and rehabilitation interventions.

For clinical trials aimed at motor restoration, outcome measurement tools should be relevant to the anticipated targets of repair-enhanced rehabilitation. Most outcomes to date have been drawn from disease-specific and rehabilitation toolboxes. In studies that include participants who are more than a few weeks beyond acquiring profound impairments and disabilities, outcome measures will likely have to go beyond off-the-shelf tools that were not designed to detect modest clinical evidence of sensorimotor system repair. This chapter describes specific rehabilitation strategies and outcome assessments in the context of interfacing them with neurorestoration approaches.

Keywords Stroke • Spinal cord injury • Traumatic brain injury • Rehabilitation • Neuroplasticity • Motor learning • Robotics • Skills practice • Noninvasive brain stimulation • Outcomes

Other chapters in this text describe novel molecular, cellular, and pharmacological approaches that may be applied to try to improve outcomes in persons with disabling stroke and neurotrauma. Here, we will concentrate on augmenting these

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approaches by neurorehabilitation interventions, primarily for studies that aim to improve profoundly impaired motor control. Indeed, one might say that the novel approaches really should be considered as methods that aim to augment rehabilitation targeted to the sensorimotor system.

Much work and money goes into preclinical experiments to generate, for example, a reproducible and safe cell type and method of delivery, as well as establish possible mechanisms of action that are associated with chosen outcomes in a model of stroke, traumatic brain injury (TBI), or spinal cord injury (SCI). When the clinical trials are planned, however, biopharma and the U.S. Food and Drug Administration (FDA) may not want to include rehabilitation therapies. Their concern is that this combination becomes a test of two different interventions at the same time, so distinguishing adverse responses and positive outcomes related to each may be difficult. More likely, however, targeted rehabilitation, through mechanisms of activity-dependent plasticity, will maximize the potential efficacy of these novel biological approaches.

On the other hand, since neurorestoration is the goal, clinical endpoints for trials may be recommended by the FDA that primarily include off-the-shelf measurement tools that were not designed for neural repair outcomes. For acute interventions for stroke and TBI, carried out within the first 2–3 weeks after onset, conventional rehabilitation therapies and clinical measures may not need major modifications. But to promote and measure gains in studies of subacute or chronic interventions for profound and presumably minimally changing impairments, more specific strategies for rehabilitation protocols and relevant outcome measures should be considered.

This chapter emphasizes approaches that may be undertaken for more severely impaired subjects whose predicted level of future function is otherwise low. These participants in a trial might have no functional use of an upper extremity or be able to walk without human assistance.

Substrates for Rehabilitation Strategies

The neural substrates for recovery, often described as mechanisms of neuroplasticity, exist within spared neural pathways and compensatory neural and behavioral adaptations. Rehabilitation takes advantage of fundamental features of neural circuits, which include the capacity to make molecular, structural, and physiological changes within and across neurons, axons, dendrites, glia, and synapses in response to experience, training, and learning. The underpinnings of neurorehabilitation have been established in animal studies of normal mechanisms of skills learning, effects of enriched environments, behavioral experience, and postinjury training that remodels neural networks at multiple levels of the neuroaxis [1]. Indeed, training and enriched environments are increasingly included in preclinical and occasional clinical protocols for repair [2, 3]. Training, exercise, and biological processes for axonal regeneration, dendritic sprouting, and neurogenesis are inherently interactive. The success of remodeling and of strengthening neural connections in humans will,

based on animal models, also depend in part on the timing of the intervention postinjury [4, 5] as well as the reproducibility of repair responses from experimental models to patients, lesion size and location, the lesion's milieu of pro and antiregenerative molecules and physical barriers, age, premorbid skills and cognitive strengths, experience since onset of injury, medications, comorbid diseases, etc. [6, 7].

The sensorimotor networks engaged in improving performance and consolidating skills in patients are also highly integrated with other systems that represent components of cognition, including working memory, executive functions, error and novelty detection, reward and motivation, perception and imagery, responsiveness to verbal and physical cues during training, and language. Cognitive impairments are common after stroke from focal lesions, prior subcortical white matter lesions, and aging. They are especially prominent after TBI with diffuse axonal injury; many spinal cord injuries are also accompanied by TBI. This degradation in connected and remote networks may have to be addressed by rehabilitation to maximize improvements in motor skills and the ability to participate in usual personal and social activities [8]. That therapy, however, may add to the complexity of a neurorestoration clinical trial. For example, if not an exclusion criteria, aphasia, impaired working memory, impaired planning, hemi-neglect, and hemianopia may interfere with motor-related rehabilitation and the process of measuring outcomes. Some evidence-based interventions exist for certain cognitive impairments, but most are less well tested than motor skills training in chronically impaired patients [9–12]. On the other hand, spared domain-general, nonmotor networks, as well as contralesional motor regions, may be overactive as patients try with effort to accomplish a task [13–16]. Modulating these regions by physical or cognitive therapies or direct cortical stimulation may contribute to rehabilitation gains [17]. The clinical examination, along with structural and functional imaging with activation and connectivity studies, can help determine the integrity of diverse networks and their adaptations over the course of interventions.

Initial trials of cellular, molecular, and novel pharmacological approaches for stroke, TBI, and SCI seem most likely to try to improve the function of a highly paretic or plegic upper extremity (UE). That need not be the only goal of the trial, but it is one for which many rehabilitation strategies have evolved to achieve an important aspect of quality of life. Participants are likely to have a highly impaired arm and hand, probably with little or no selective movement against gravity at the wrist and fingers after supraspinal lesions. On the Fugl-Meyer Assessment Scale, they might score around 15–25/66. Other participants are likely to have no movement one level below a cervical SCI. The goals of the biological approach with rehabilitation may include functional reaching, gripping, pinching, and using the UE for tasks within one's peri-personal space to eat, groom, and assist other valued tasks.

The science of biological approaches will benefit from any demonstration of restoration, such as producing movement of the wrist and fingers against gravity at one or more joints when none had existed at baseline. The participants, however, may not benefit in their daily activities if they do not regain reach, grasp and release to hold and manipulate objects or the ability to walk. An intervention that carries risk, such as invasive procedures to implant cells, must ultimately enable useful

new function. Outcome measures, however, that can detect less than functional sensorimotor gains are critical to future advancements. Rehabilitation strategies can help promote this goal.

Neurorehabilitation Strategies

Rehabilitation is usually a multidisciplinary team effort led by a neurologist or physiatrist. That team might consider, for the individual participant in a trial, ways to quickly neutralize or reverse impairments that may interfere with the goals of the biological approach. This might include managing modest contractures, hypertonicity, muscle atrophy and disuse weakness, deconditioning, pain in joints from overuse, depression, anxiety, medications that may interfere with the actions of the biological approach or with learning and attention, and modest cognitive and perceptual impairments that interfere with daily activities.

Specific rehabilitation strategies to improve motor-related functions have common denominators after stroke, SCI, and TBI, but are primarily effective for persons with mild to moderate impairments [18]. These strategies usually include progressively more challenging task-related practice, repetition with feedback about aspects of performance using physical and verbal cues, and meaningful goal setting. Table 28.1 lists basic rehabilitation strategies and more experimental ones that may find a role, depending on the targeted impairment of the novel repair approach. When applied to participants in near future trials of neurorestoration, some of these methods are likely to interact iteratively with molecular, cellular, and pharmacological approaches to help activate or disinhibit a relevant neural network, alter the molecular milieu to better enable regeneration and synaptogenesis, and help sculpt selective recovery of movement. Thus, it is not enough to simply record whether any physical, occupational, or language/cognitive therapy was provided and its duration. Leaving the type, intensity, and duration of therapy open-ended and uncontrolled may introduce noise that a covariate statistical method cannot correct. Therapy ought to be standardized and optimized to improve targeted sensorimotor outcomes in the experimental and control arms of a trial.

Strengthening and Aerobic Fitness Exercise

Exercise has many effects on genes and molecular cascades that have been associated with learning, memory, and regeneration [19, 20]. Deconditioning and disuse muscle weakness can impede functional activities. A baseline level of aerobic and strengthening exercises ought to accompany biological interventions in highly impaired participants. Isometric, eccentric, and concentric exercise can be used to strengthen muscle groups that may contribute to a newly organized movement. Even a modest increase in agonist or antagonist power may enable a newly evolving movement to reach a clinical threshold, if the biological intervention is successful.

Table 28.1 Rehabilitation approaches for trials that can be combined to augment biological strategies to regain motor control of upper or lower extremities after stroke, TBI, and SCI

Basic strategies
Coordinated, multidisciplinary rehabilitation team care
Progressive intensive practice
Targeted impairment-related practice
Targeted task-oriented practice
Constraint-induced movement therapy practice protocol
Over-ground training of walking and balance with corrections for temporospatial, kinematic, and kinetic deviations
Body weight-supported treadmill training
Exercise for general strengthening and fitness
Walking aids, splints, orthotics
Cognitive training for impairments in sensorimotor integration, attention, working memory, and executive function
Wearable sensor-derived feedback about type, quantity, and quality of exercise and skills practice; motion algorithms to remotely monitor practice
Smartphone cueing to encourage practice sessions
Tele-rehabilitation to monitor and progress home-based practice and functional changes
Possibly useful strategies
Action observation; mental practice by motor imagery
Bimanual UE practice
Biofeedback of force, direction, angle, and speed of movements
Brain–machine interfaces to augment network feedback in training
Electrical stimulation with or without EMG feedback of targeted muscle groups
Electromechanical or passively supportive exoskeletal assists
Functional electrical stimulation of components of sought movement
Neural prostheses to enable training
Noninvasive brain stimulation (NIBS) of a specific network during practice
Transcranial magnetic stimulation
Transcranial direct current stimulation
Peripheral nerve and somatosensory stimulation
Pharmacologic modulation of neurotransmitters and learning modulators
Robotic-assisted movement training with feedback
Spinal cord electrical stimulation to lower the excitability threshold of motor pools
Virtual reality computer and immersive training environments

Task-Oriented Training

Progressively challenging practice of selective voluntary movements, initially supported by a therapist or caregiver, can lessen moderate chronic impairments and disability, as well as contribute to gains early after injury [21]. Practice ought to be goal-oriented and relevant to personal goals for skills retraining. In general, no one therapy listed in Table 28.1 is clearly better than another, but many have revealed efficacy compared to no specific intervention. For example,

constraint-induced movement therapy (CIMT) has received much attention. The Extremity Constraint Induced Therapy Evaluation (EXCITE) trial showed that 10 full-day sessions over 2 weeks with 60 or more hours of upper extremity practice that increasingly shaped more complex movements in the hemiparetic arm, plus about 6 h per day of forced use at home by gloving the unaffected hand, led to better function of the arm and hand compared to no therapy in patients who were 3–9 months after stroke [22]. Candidates for CIMT, however, must already have at least 10° of wrist and finger extension, which suggests a fair level of motor control. Without some wrist and hand function and ability to reach, constraint of the unaffected hand would not be feasible at home. The value of the intervention is that it includes a range of progressively difficult UE practice movements across single and multiple joints and real-world tasks, in keeping with other task-related, repetitive practice paradigms for motor skills learning. However, even 2 h of progressively challenging therapy with little or no constraint also seems better than less focused UE therapy [23].

Splints and orthotics may better position a joint so that newly acquired movements can be practiced and made more functional. For example, an orthotic that slightly extended the paretic wrist might enable active pinching if modest finger extension and flexion recovered. For a trial of a biological approach, the investigators ought to specify what orthotic was needed and what function was gained by making it available.

Robotic-Assisted Upper Extremity Training

Some cleverly designed electromechanical-assistive devices such as shoulder–elbow–wrist controllers have undergone clinical trial testing. The results, in general, especially for highly impaired participants after stroke and SCI, are generally not better than more conventional training techniques [24, 25]. The Veterans' Administration's upper extremity robotics trial offers some insight into expected outcomes for highly impaired hemiplegic persons [26]. The entry criteria was moderate to severe motor impairment, defined as a score of 7–38 on the Fugl-Meyer Motor Assessment of an upper limb from a stroke that had occurred at least 6 months before enrollment. At 12 weeks, the mean Fugl-Meyer score for patients receiving robot-assisted therapy was better than that for patients receiving usual care, meaning no intervention, by 2.17 points and worse than that for patients receiving intensive conventional rehabilitation by −0.14 points, but the differences are rather trivial and not statistically significant. This study may represent the maximum gain for an UE skills training protocol for the types of patients likely to be tested with cellular therapies, at least that can be measured by the Fugl-Meyer, which looks at a series of synergistic and more selective movements, However, the use of such robotic devices for Phase II and III trials of novel biological interventions could enable a reproducible rehabilitation strategy for highly impaired participants.

Mobility Training

Early biological trials are likely to include the goal of reciprocal leg movements and balance for walking after stroke and SCI. Participants at time of entry are likely to be unable to flex at the hip or extend the lower leg against gravity [27–29]. Progressive practice over ground includes selective muscle strengthening, building endurance, and physical and verbal cues to improve spatiotemporal, kinematic, and kinetic aspects of reciprocal leg movements and balance for walking. Goals include aiming to lessen asymmetries between the legs in single-limb stance and swing duration, and increase stride length, speed, and distance walked with or without passive assistive devices. These goals have been addressed, along with enhancing fitness, using body weight-supported treadmill training and robotic-assistive electromechanical devices. The results suggest that these interventions do not improve walking-related outcomes more than conventional gait training over ground of equal intensity after disabling stroke [30], SCI [31, 32], or TBI, but these strategies may enable step training and trunk strengthening in highly impaired subjects to facilitate the potential effects of a biological approach [33]. Intelligent exoskeletons for walking practice may also serve as training devices—several commercial ones are now available to enable stepping over ground.

Noninvasive Brain Stimulation

Much recent research has examined the potential for transcranial direct current stimulation (tDCS) [34] and repetitive transcranial magnetic stimulation (rTMS) [35] to improve motor function after stroke, especially for UE and swallowing movements. The data suggest that the best results come from a combination of targeted practice during the time of brain stimulation, which may unmask latent pathways, strengthen residual and new connections, modulate neural oscillations, and potentially increase functional connectivity [36]. However, the modest gains found so far apply only to patients with mild to moderate motor impairments.

Repetitive TMS studies to date use highly variable stimulation protocols and assessments of outcomes. If rTMS is used to try to augment biological repair along with rehabilitation, further experimentation will be necessary to determine whether to directly excite ipsilesional primary motor cortex (M1) or another motor-associated region; indirectly excite ipsilesional M1 by suppression of contralateral M1; optimize the type and frequency of stimulation such as theta burst, 1 or 5 Hz stimulation which have very different short-term physiological effects; carry out a simple attentional or targeted muscle contraction [37] or a more skilled task during and for a short time after the stimulation protocol; understand what aspects of a movement may benefit from any sort of stimulation; optimize the number and schedule of bouts of stimulation plus therapy; or continue to train beyond the time of stimulation. For some repair strategies, rTMS and tDCS may be able to augment descending drive to uncrossed and recrossing corticospinal and other supraspinal axons that

activate motor pools for selective and combinational movements. It is most intriguing that cortical electrical stimulation may increase sprouting of the unaffected corticospinal tract onto the ipsilesional ventral horn of the spinal cord [38].

Other CNS and PNS Stimulation Adjuncts

Other electrical means to increase excitability of latent residual pathways may be of interest in biological trials. Methods include continuous deep brain, direct spinal cord, and peripheral nerve stimulation during practice [39]. Deep brain stimulation to date is probably too invasive to serve as an adjunct—methods to modulate neural oscillations would have to be shown to be efficacious by independent trials. A single-subject design of spinal cord stimulation in motor complete paraplegic participants enabled modest voluntary leg movements, sometimes against gravity. Perhaps the stimulation lowered the threshold for motor neuron excitability by latent supraspinal inputs to them [40]. This does not imply that the subjects would be able to walk, however. But if a less invasive stimulation intervention proved feasible and reproduced such findings, then it might augment the use of biological approaches to provide circuit specificity for further training. Pairing TMS with peripheral nerve stimulation and dual bihemisphere TMS may also selectively increase cortical network excitability to augment training, but efficacy studies are pending.

Brain–Machine Interfaces

A brain–machine interface (BMI) [41] for rehabilitation uses an analysis of various types of brain signals from imagining a movement to direct the desired movements of, for example, a robotic arm. This training may augment synaptic efficacy for the actions performed and drive latent pathways that can come to be involved in solving the movement problem. Early studies suggest that practice, combined with cortically implanted electrodes and robust movement-associated algorithms, can improve motor control, leading to improvements in functional connectivity of motor-related pathways [42]. Affordable, safe, and efficacious complete systems for rehabilitation to try to improve motor control of a plegic limb might complement an intervention for neural repair.

Other Possibly Complementary Interventions

Electromyographic feedback from a minimal voluntary muscle contraction that then triggers functional electrical stimulation to increase the contraction has improved the voluntary control of single muscle groups and may be useful when the repair strategy aims to increase supraspinal control of that muscle [43]. This may be most applicable to the patient with a cervical SCI who is trying to regain motor control

1–2 levels below the lesion or in the hemiplegic patient trying to regain wrist or finger extension.

Many other techniques may serve to help engage, activate, and reinforce a neural network to focus neural resources on accomplishing a sensorimotor task. Training in a virtual reality environment, using imagery of a task as a form of practice, and UE mirror therapy have been of some benefit in patients with fair motor control [44–46]. All increase activation of M1 and other cortical and subcortical motor network nodes [47]. These are potential adjuncts for biological approaches, but may be difficult to incorporate into Phase II or III trial designs because they add complexity.

Pharmacologic Agents

Medications developed for other uses, especially ones that may act as neurotransmitters and on attention, have a long history of being tried for stroke and TBI. None so far have enough evidence behind them to warrant use as an adjunct in a repair trial. The most likely to be considered would be fluoxetine [48], reboxetine [49], and amantadine, but not dopamine agonists [50]. For cognitive and behavioral outcomes, modest if any benefits are apparent for cholinergic and catecholinergic drugs that might also impact motor control [51].

Tele-rehabilitation

The field of mobile and wireless health (mHealth) [52] offers ways to monitor, remotely and inexpensively, the activities of participants in trials. Wearable wireless sensors, such as accelerometers and gyroscopes, can recognize the type, quantity, and quality of walking, cycling, running, leg exercises, and other nonsedentary behaviors by fusing signals from the legs and analyzing them with pattern recognition algorithms [53]. Thus, it should be feasible to monitor how much and how well a trial participant is practicing a rehabilitation strategy, give verbal or text feedback about performance over a smartphone, and collect interim ratio scale measures relevant to outcomes and adverse responses. This scenario may enable more subjects from remote geographical regions to conveniently enter trials and limit the burden of repeated clinic visits. Serial monitoring and objective sensor-based annotation of targeted movements may also enable investigators to better discern between restoration versus substitution versus compensation within changes in functional movement goals [54].

Combinational Strategies

The combination of a molecular, cellular, or novel pharmacological approach with targeted rehabilitation would seem likely to augment each other and increase the likelihood of more robust outcomes. This is one of many enrichment strategies for

Phase II and III trials [55]. Is there a cost-effective way to interleave rehabilitation with a biological approach during a randomized clinical trial?

The STEPS participants recommended that cellular therapy trials should include at least two pretreatment baseline examinations to assure a stable baseline in a homogeneous group of subjects [56]. For trials that start in a late subacute or chronic period after injury onset, however, spontaneous degradation of function may have intervened or latent function may not be brought out by the neurological examination. One solution is to phase in therapy for targeted improvements for 10–12 sessions for 2 h each over 2–4 weeks, focused on, for example, UE motor activities, emphasizing the shaping and progressive practice procedures used in the EXCITE trial [55]. This training might include the use of a resistance stretch band for strengthening exercises, if feasible. If the repeated neurologic examination and primary outcome measurements are stable, the investigators can proceed with the biological intervention with greater confidence that any gains can be attributed to the experimental intervention. Concern about forced or early high levels of exercise has been raised by studies in animal models [57]. However, this may be more of an issue within the first 3–7 days after onset of injury in animal models, rather than in clinical trials, where intensive exercise falls far below what mice and rats can be induced to do.

A phase-in of therapy also reinforces how to practice. Further practice can be accomplished at home using wearable sensors or a tele-rehabilitation protocol to encourage and monitor practice. Every 1–2 weeks, a centrally located therapist can watch the subject at practice using a smartphone or tablet camera, review summarized sensor data about daily activity, and make suggestions about how to continue. Possible advantages to this scenario are that the trialists will annotate the therapy actually received, improve reliability of procedures, develop dose–response information regarding motor changes over time, maximize the interaction between the biological intervention and rehabilitation, and generally increase the validity of the trial. This strategy may also provide the basis to improve future trial designs as well as test new sensor-based outcome measurement tools.

Outcomes

The STEPS participants suggested the potential use of modality-specific outcome measures, tested in a Phase II design and possibly serving as the primary outcome in a Phase III cellular trial [56]. This approach could lead to a modality-specific FDA label for the approach, but that may be fine for a study of motor recovery [58].

The combination of a biological approach with targeted rehabilitation lends itself to developing the outcome measures that are most likely to be driven by the combination. What is practiced should have a close relationship to the primary outcome measurement. Rehabilitation plus repair also represents a complex intervention. For trials, the investigators will want complex outcome measures, so they can detect (1) any biological activity of the repair intervention; (2) change in impairment;

(3) any clinically meaningful increase in daily functioning and participation in relevant activities; and (4) self-reported change in quality of life for better or worse. Biomarkers of repair such as functional, connectivity, and structural MRI and perhaps TMS for changes in cortically elicited motor evoked potentials may provide other ways to detect motor responses to the interventions.

Many of the varied symptoms, impairments, and functional activities of patients may change to differing degrees over the course of a biological intervention. It is costly and a burden on participants to try to measure everything, looking for a sign of improvement in neural functioning. If a nonmotor outcome is of interest, however, a baseline level of function will be necessary. For example, if improved bladder control is a possibility, i.e., continence, voluntary voiding, no retention, etc., then several weeks of measures of urine frequency and post-void residual volumes are needed as a measurement tool for comparison in a secondary analysis. After a high SCI, if dysautonomia is targeted, then delete, prebiological therapy for blood pressure and heart rate, spasms, and bouts of dysreflexia, as well as symptoms, must be serially monitored for several weeks before and after the treatment.

NIH-funded trials ought to include standard measures that allow comparisons across trials, such as those described in the NIH Toolbox. But the FDA and biotechnology companies ought to consider the likelihood that such tools may not capture the proof of principle about whether a cellular intervention modulated biological activity in ways that fell below the sensitivity of those standard tools. Consider the ordinal-scaled stroke tools, such as the NIH Stroke Scale, modified Rankin Scale, and Fugl-Meyer Motor Assessment. The NIHSS looks only at gross sensorimotor impairment. The Rankin emphasizes walking ability with a mix of impairment and disability categories, but does not provide any standard way to assess the details of motor functions and motor- or cognitive-related disability. The Fugl-Meyer assesses limb movements in and out of upper motor neuron synergies. The scale cannot assess more subtle single joint motor changes, so it is generally not a targeted outcome measure. Another commonly employed tool is the American Spinal Injury Association AIS Impairment Scale for sensorimotor testing. Only one muscle is tested for each of the C4–T1 and L2–S1 root innervations, so changes in other groups may go undetected. TBI measures tend to underemphasize functional movements in favor of cognitive and participation scales. Phase II trials could include potentially more sensitive outcome measurements that are specific to anticipated motor changes, as well as assess-related functional gains (Table 28.2).

Table 28.2 Protocol for weaving a cellular, molecular, or novel pharmacological intervention with rehabilitation for a motor deficit

1.	Initiate a rehabilitation strategy that is relevant to the anticipated outcomes for the biological intervention
2.	Continue until a stable within-subject baseline is achieved for anticipated motor outcomes
3.	Initiate the biological intervention
4.	Depending on preclinical and prior clinical dose–response studies, restart a similar progressive rehabilitation strategy within the best timeframe in both experimental and control groups
5.	Serially measure the primary outcomes for the biological and rehabilitation interventions that are being studied

Motor assessments might include testing 3–4 muscle groups from each root level for the arm and leg, using the British Medical Council Scale. Where voluntary movement was ≤3/5 before the intervention, the joint should be positioned on a fixed surface to detect new degrees of movement. Surface electromyography and wireless sensors such as accelerometers, gyroscopes, and goniometers may be applicable as monitoring tools for newly organized movements. Scales such as the Fugl-Meyer for selective multijoint movements would supplement the targeted decrease in motor impairment, as would timed tasks and functional scales that were relevant to the goals of the rehabilitation plus biological approach.

Conclusion

In testing molecular, cellular, and novel pharmacological restorative approaches, rehabilitation skills training should aim to optimize improvements in targeted sensorimotor outcomes, as well as other goals for impairment, disability, and participation. This dual strategy may selectively activate neural networks to optimize connectivity, learning, and memory. Outcome measurement tools need to be sensitive enough to describe and quantify newly induced improvements.

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Chapter 29
Bridging the Chasm Between Scientific Discovery and a Pivotal Clinical Trial for a CNS Disorder: A Checklist

John D. Steeves

Abstract The central nervous system (CNS) is difficult to treat effectively after damage, whether the situation is congenital, traumatic, or degenerative. The effective translation of a novel preclinical discovery to a clinically meaningful human treatment is demanding and initially governed by fundamental achievements at the preclinical development level. Good laboratory practices (GLPs) are increasingly being adopted, as they provide all neurological investigators with increased confidence for the results. GLPs are demanding and ask scientists to adhere to many of the demanding criteria intrinsic to human studies. The subsequent preclinical development of a therapeutic is equally important and outlines the safety, dose, fate, window of opportunity, and route of administration.

Human trials are channeled by established guidelines, but CNS clinical studies involve target populations that are heterogeneous and often rely on subjective (ordinal) outcome tools that can be questioned for their ability to accurately and sensitively discern subtle treatment effects. Improved solutions for the following concerns are evolving quickly:

1. What is the most appropriate type of participant to enroll in each phase of a trial program?
2. What would be the most accurate, sensitive, and reliable outcome measure for the chosen clinical target?
3. How is a clinical endpoint threshold selected to determine whether the therapeutic provides a meaningful clinical benefit?

Keywords Good laboratory practice • Therapeutic development • Human study • Subject heterogeneity • Clinical outcome measures • Stroke • Spinal cord injury

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Challenges Common to CNS Disorders

Almost everyone would agree that the central nervous system (CNS) is the most difficult organ of the body to treat effectively after damage, whether the situation is congenital, traumatic, or degenerative. It is impossible to cover all aspects in the translational process of an experimental therapeutic for every CNS disorder. Therefore, I will focus on aspects that are most common to all. Below are "10" inescapable facts about CNS disorders:

- Most CNS disorders are complex and result in heterogeneous groups of symptoms (syndromes) with varying spontaneous outcomes (recovery or deterioration).
- Adequately developing a scientific discovery from "bench to bedside" is challenging and often only partially completed before an experimental treatment enters human study.
- Patient transport to a study center and the necessary screening for the recruitment, consent, and enrolment of study participants takes time to complete (often a minimum of 6–12 h). This limits the window of opportunity for neuroprotective interventions.
- The blood–brain barrier (BBB) can limit penetration of some therapeutic treatments from the circulation, presenting an impediment to easy systemic administration.
- Heterogeneity in study participant characteristics should be minimized, but is often difficult to determine at early time points after the onset of a CNS disorder.
- Meaningful functional improvement is the necessary pivotal trial endpoint; imaging and biomarkers are useful, but only for establishing and tracking biological efficacy.
- Current therapeutic strategies have not and should not be expected to provide large treatment effects in the near future. Thus sensitive, accurate, and reliable outcome measurements are needed to detect subtle therapeutic benefits.
- Development of clinical outcome measurement tools and trial endpoints (treatment thresholds) requires thoughtful selection based on rigorous clinimetric modeling.
- Regulators, investors, health care payers, and participants may not always have similar expectations for the outcomes of each phase of a clinical trial program.
- All evidence suggests that combinatorial treatments will be needed to provide the greatest functional benefit in a clinically meaningful manner.

It is not unreasonable for you (the reader) to think the challenges are too overwhelming and want to quit reading at this point. However, the neurological sciences have learned from past experiences and errors. There have been a number of recent advances using more sophisticated approaches for the development of improved study protocols to more accurately validate CNS therapeutic interventions. Furthermore, the outcomes from a number of recently developed interventions appear to be providing subtle improved benefits.

Preclinical Validation Prior to Entering Human Study

The presumption here is a discovery of a therapeutic intervention has already been achieved in a preclinical animal model and it is thought that this discovery could have utility for a human disorder. Furthermore, the therapeutic can be administered within a clinically relevant time frame (window of opportunity for humans) for acute CNS disorders. Any experimental treatment that must be administered within a few hours after the onset of an experiential CNS disorder is not likely to be clinically relevant, as it will not be provided within the available time frame for effectiveness.

Unless we change study consent rules and allow paramedics to routinely administer experimental treatments, this means the early events associated with acute secondary cell damage are likely to remain difficult therapeutic targets. Even if such a strategy was deemed safe for drug administration, the heterogeneity of the patient population does not guarantee a positive benefit. The recent Field Administration of Stroke Therapy–Magnesium (FAST–MAG) study [1] found that magnesium sulfate could be safely administered by paramedics (<2 h after stroke onset) while transporting stroke patients to hospital. However, there was no evidence for improved outcomes, 90 days later, when compared to placebo controls.

First and foremost, and said by many, it is optimal to have a scientific discovery validated by independent investigators (e.g., [2, 3]). Using appropriate animal models, preclinical validation might involve several different forms of confirmation, including:

- Using the methodology of the original publication (detailed research methods should be provided by the initial investigators).
- Using small variations or improvements in the treatment protocol (demonstrates robustness of the initial discovery).
- Using different animal species, with at least one genus being an outbred species (demonstrates the fundamental biological nature of the target or intervention).
- Using a preclinical outcome measure that is clinically appropriate to the human CNS disorder (predicts scientific discovery might have similar clinical benefit).

Successfully accomplishing all or some of the above validation studies will provide increased assurance to the field that the preclinical treatment is worth pursuing. Not all of the above validation attempts will necessarily be completed. There are some significant disincentives to scientists replicating a scientific finding. With some justification, it is often perceived that confirming another scientist's discovery is not very meaningful to career progress. Second, performing the same methodology can be difficult, as the most arcane change in technique can lead to different results. Such nuanced or overlooked alterations in methodology are usually unintentional, but might argue that the experimental treatment will not be very robust in its potential application. Third, proprietary interests associated with intellectual property development and investor funding can block validation attempts, unless the second scientific group is willing to abide by the nondisclosure agreements that are likely to impede rapid publication in a peer-reviewed journal.

Good Laboratory Practice Reduces Experimental Bias

It is important to protect against unintended bias influencing the conduct or analysis of preclinical experiments. In 1999, The Stroke Therapy Academic Industry Roundtable (STAIR) was concerned about the failure of a large number of stroke trials. STAIR (www.thestair.org) introduced a series of guidelines to improve experimental design in preclinical studies to reduce bias and increase the confidence for translating any observed neuroprotective efficacy in animal models to the more heterogeneous circumstances of human stroke. The STAIR guidelines established "good laboratory practice" (GLP) and if followed, it would reduce the possibility of bias influencing any reported outcomes. GLP requires voluntary compliance by investigators, as there is no regulatory oversight. It also requires an individual or group of individuals, not directly involved in the study, to independently oversee and conceal records of randomization, study group identity, allocation sequence, and any removal of an animal from a study (see below).

In scientific experiments, *confirmation bias* is a tendency for people to confirm their preconceptions or hypotheses, independent of whether they are true. Scientists are as human as anyone and their devotion to a preconceived idea can lead them to see a positive result when none exists. A second type of bias is *selection bias*, which is the tendency to publish the desired outcome, without mention of the number of animals excluded from analysis (which presumably did not show the desired result). Bias is also something that could influence clinical trials and the *CON*solidated *S*tandards *o*f *R*eporting *T*rials (CONSORT) initiative has led to improved clinical trial practices and reporting (see below).

Some of the key GLP elements have been outlined [4] and they include the following:

- Precise details of the animal models used, including any genetic modifications.
- The method for randomizing animals to the experimental and control group should be stated and the identity to which group the animal was assigned must be kept from any investigator providing treatment or assessing outcomes, until the analysis of the results is complete.
- The number of animals removed from the study or excluded from analysis should be reported. There are good reasons to exclude an animal (e.g., poor health or pain), but the criteria should be determined in advance and removal of an animal should occur without knowledge of which study group the animal was assigned (experimental or control).
- Prospectively describe the expected difference between the experimental and control groups, as well as complete sample size calculations. During analysis, use more rigorous statistical criteria such as standard deviation, instead of standard error of the mean.
- Prospectively describe the inclusion and exclusion criteria for the experiment, including criteria being used to establish the severity of the symptoms characterizing the induced experimental deficit.

- Always use "blinded" assessments of experimental outcomes and carefully describe these assessment procedures. There is no need for the principal investigators to conduct subjective behavioral assessments as these can and have been accurately undertaken by trained individuals (often an undergraduate student volunteer).
- In the case of dose response comparisons, the allocation sequence must also be randomized and concealed from everyone involved in the treatment administration, care of animals, or assessment of outcomes.
- Finally, any relationship that could be perceived a potential conflict of interest or absence of any such relationship must be disclosed and acknowledged.

Preclinical Therapeutic Development After Validation of Experimental Efficacy

There are additional aspects of a preclinical development program that must also be completed prior to moving to human study [2, 3], including establishing:

- Safety (adverse event and side effects) in more than one animal species, as safety of the experimental treatment is paramount to any translational process. Confirming the maximal tolerable therapeutic dose in more than one animal species is fundamental. Careful documentation of any adverse events, as well as the possible toxic profile of a drug or cell transplant (e.g., activation of neuropathic pain or the formation of tumors).
- "Window of opportunity" for benefit (i.e., when must the treatment be provided in relation to the initial onset of the CNS disorder). As outlined above, a short-time window will limit clinical application. Considering the time for patient transport, the accurate diagnosis and stabilization of the patient for possible treatment, as well as any transfer from a community hospital to a level-one trauma center with the appropriate study resources may take one or more days. This will limit recruitment and enrolment.
- Formulation of the therapy is critical, not only to IP rights, but also to the design of the most clinically feasible form of the therapy to be applied to human subjects. As an example, understanding the structure of a pharmaceutical can enable combinatorial chemistry to generate improved formulations of a drug (e.g., sustained release forms and/or remove unwanted side effects).
- Route of administration changes the invasiveness of a therapeutic application. Oral or systemic administration (e.g., intravenous) is less invasive and easier to implement than a treatment requiring a surgical intervention for delivery. However, given the efficiency of the human immune system and the effectiveness of the blood–brain barrier, oral or systemic application is not always possible for a CNS disorder. This does not just apply to drugs and cell transplants, as some assistive devices require surgical implantation (e.g., epidural infusion pumps, brain–computer interfaces).

- Dose scaling (from small to large species) is a difficult endeavor requiring allometric scaling for size differences, but also for size-independent variables [5]. Allometric scaling can be more sensitive when pharmacokinetic parameters, between species, are considered, such as differences in drug liberation (from a carrier vehicle), absorption, distribution, metabolism, and excretion (often abbreviated as LADME). Interspecies differences can alter one or more of the LADME characteristics and dramatically change the effective dose of a drug or cell transplant.
- Fate of the administered therapeutic is critical to establishing an effective dose range for a therapeutic and relies on the well-established principles of pharmacokinetics (i.e., what the body does to a drug, cell, or device) and pharmacodynamics (what the drug, cell or device does to the body). The LADME principles of drug pharmacokinetics have achieved a richer history of understanding than what is understood for the fate of cells after CCS transplantation.

In general, we would like to know more about where transplanted cells go after infusion (i.e., migration), whether they survive long term or are phagocytized and removed from the CNS [6, 7]. We are slowly gathering information about the influence of the host tissue to stimulate or limit in vivo phenotypic differentiation. On the "cellular pharmacodynamics" side of the equation, we need to understand what cellular signals are released by transplanted cells into the host tissue, as well as how cell transplants interact or alter host cells and hopefully it does not include carcinogenic properties. Likewise, any implanted CNS device is likely to have a "*yin and yang*" interaction with the host tissue where the benefits of the device's actions must be weighed against possible adverse events such as tissue damage by the device or the inactivation of the device by deposition of excessive connective tissue (fibrosis).

- Mechanism of action is not always essential, as many current clinically beneficial therapies are phenomenological, but it can certainly help guide the development of the "next generation" of a therapeutic, as well as direct the processing of new formulations or treatment options.
- Finding a certified and scalable "good manufacturing process" (GMP) facility for fabricating the therapeutic may appear to be a secondary matter. However, if after a Phase III trial, the experimental therapeutic (e.g., drug or cell-line) is approved as a treatment option for the disorder and the GMP facility lacks the capacity to manufacture sufficient quantities of the tested and validated treatment product, the regulatory agency may require another round of clinical studies to validate any "new" formulation of the product (i.e., drug batch, cell-line).

General Requirements for Clinical Trials and the Goals of Various Study Phases

Any CNS clinical study must follow the well-established principles outlined for all clinical trials, including adequate informed consent, randomization of study participants, blinded assessments of study outcomes, with no payment by subjects to participate in a study or payments to study investigators. It is essential to comply with

the latest version of the CONSORT statement (http://www.consort-statement.org), as well as the ethical principles for medical research involving human subjects (or Helsinki declaration) as endorsed by the World Medical Association (http://www.wma.net) and subsequently by all national regulatory agencies [8].

Every CNS disorder, no matter how rare or common, is heterogeneous in terms of symptoms, diagnosis, and/or prognosis [3, 9]. In fact, this statement could be made for any human malady. It is human nature to simplify descriptions. However, the inclusion of heterogeneous or inappropriate participants in a clinical trial can lead to ambiguous conclusions, as well as waste valuable resources, both human and financial. Each phase of a clinical trial program has distinct goals and consequently different parameters, protocols, outcome measures, and endpoints that govern the conduct for that stage of investigation.

Phase I trials are centered on the initial exploration of safety and in the case of a drug or cell transplant often include an evaluation of the responses to different therapeutic doses. Of course, safety is continuously monitored throughout all subsequent trial phases. Phase I safety trials are often conducted in healthy volunteers, but this is not a viable approach for cell transplant procedures. A nuance for Phase I is the recent use of a combined Phase I/IIa approach where safety data are collected along with pilot data on therapeutic activity or functional benefit. The motivation here is to advance decisions surrounding the continued funding for the trial program [2]. As Phase I studies often involve small sample sizes, such hurried approaches can lead to errors in the judgment of efficacy. In the case of spinal cord injury (SCI), participants with a sensorimotor complete thoracic SCI are often recruited to Phase I studies, specifically because any adverse neurological event associated with the experimental treatment would be less likely to impair upper extremity function in this study population. It is unfortunate, but true, that detection of any therapeutic effect in sensorimotor complete thoracic subjects is very difficult [10, 11].

Phase II or Phase IIb trials are still an exploratory study with focus on the demonstration of biological activity and/or functional benefit of the intervention. They will usually measure a number of different biological, clinical, or functional outcomes to determine which endpoint is likely to be a reliable measure in a pivotal Phase III study. The determination of a primary and clinically meaningful trial endpoint is important to any subsequent Phase III study and should not be selected without some modeling of expected spontaneous recovery rates from historical datasets. This requires an understanding of what relatively homogeneous study population within the CNS disorder might be most appropriate for enrolment in a pivotal Phase III trial (see below).

After a Phase I SCI trial, for example, a high priority SCI target is cervical SCI, both complete and incomplete [12–15]. Individuals living with cervical SCI form the largest population of people living with SCI and often have the most difficulty reintegrating back into their community and/or work environment. An additional justification for focusing on incomplete human SCI is the prevailing use of incomplete animal models of SCI when developing and validating therapeutic interventions at the preclinical stage. Nevertheless, the heterogeneity of incomplete cervical SCI requires some careful decisions in the stratification of study cohorts and selection of a pivotal trial endpoint (see below).

Phase III trials are the pivotal studies where an intervention must demonstrate benefit in a clinically meaningful manner, which is then weighed against any associated risks, before approval can be determined by the relevant regulatory body. A minimal clinically meaningful difference (MCID) for a therapeutic can be difficult to define when examining a CNS disorder [16]. At present, there are few clearly validated benchmarks for demonstrating a "subtle" change in CNS efficacy to an enhanced functional capacity [e.g., activities of daily living (ADLs)] or quality of life (QOL). It should be noted that some regulatory agencies might require two independent Phase III studies prior to considering a therapeutic for approved clinical use.

After regulatory approval and adoption of the intervention as standard clinical practice, most interventions enter a surveillance period where the greatly increased exposure of a more heterogeneous array of patients enables the detection of less frequent adverse events and may provide additional information on efficacy. At this stage, it is also possible to perform phase IV clinical studies that continue to examine additional questions of efficacy, optimal treatment protocols, and safety (e.g., interactions between drugs, cell transplants, and/or devices).

Establishing Clinical Trial Guidelines

To provide some objective assistance to what is a complex series of protocol options designed to weigh the possible risks and benefits for a human study, foundations, government agencies (e.g., Food and Drug Administration, National Institutes of Health, European Medicines Agency), or independent academic–industry–foundation roundtables have established sets of disorder-specific clinical trial guidelines. Such efforts are valuable to avoid costly mistakes in terms of the ineffective use of financial and human resources associated with clinical trials.

The aforementioned STAIR guides stroke trial development (www.thestair.org). Spinal Cord Outcomes Partnership Endeavor (SCOPE) guides SCI trials (www.scope-sci.org). The Michael J. Fox Foundation (www.michaeljfox.org) and the Movement Disorder Society (www.movementdisorders.org) have been instrumental in the coordination of clinical efforts and research for Parkinson's disease or PD. It is difficult to list any one group or foundation for MS or dementia. There are a number of national and international organizations across the globe directed to MS and dementia (Alzheimer's disease). Clinical trial guidance can even be narrowed to a specific type of treatment intervention such as the guidelines published by the International Society for Stem Cell Research (ISSCR, www.isscr.org).

All guidelines undergo ongoing review and are continuously being updated with new information through peer-reviewed publications. People surviving with a neurological disorder are also becoming increasingly involved in such activities and this is important with the recent inclusion of patient reported outcomes (PROs) as clinical outcome measures.

As one example, an initial set of SCI clinical trial guidelines was developed and published in 2007 by an international panel of scientists and clinicians. This series of papers detailed the degree of spontaneous recovery after SCI [17], outlined approaches for trial outcome measures [18], discussed inclusion/exclusion criteria and ethics [8], as well as outlined various trial designs and protocols [19]. In addition, the same authors created a document written for the general public and allied health care professionals (titled: "Experimental Treatments for Spinal Cord Injuries: What you should know if you are considering participation in a clinical trial" and this is freely available at ICORD (www.icord.org) and several other SCI websites. SCOPE has since gone on to develop more detailed protocols and outcome measures for SCI.

Consideration in Planning a Clinical Trial Program

Level one evidence for the clinical efficacy of an experimental treatment is best provided by a randomized controlled trial (RCT) where participants, after assessment for eligibility and recruitment, are randomly allocated to one of the different treatment groups (e.g., experimental treatment versus placebo control). If there is a gold standard treatment available for the disorder (e.g., L-Dopa for Parkinson's disease), then the trial might compare the novel treatment against the currently available "positive control" treatment. If multiple doses of the experimental treatment are being assessed, there may be more than one experimental treatment arm [19].

Importantly, it has been argued that sham surgery controls for an intraparenchymal CNS cell transplant trial may be unethical, as they involve unnecessary surgery and therefore risk the health of control participants, however, slim. In such situations, a placebo control group may be unavailable, but every effort should still be maintained to have outcome assessments completed by observers who are "blinded" to whether the participant has received an experimental cell transplant or is a member of the untreated control group [8]. Of course this may require similar bandaging of all participants to hide any surgical scars of treatment group participants. It certainly takes the trial away from a double-blinded situation (i.e., both participant and investigator being blinded to treatment status). In such a single-blinded situation, the experimental treatment group is aware of their status and it is critical that they and the control participants not disclose treatment position to any person assessing their functional outcomes. Should this happen, a new "blinded" assessor must be recruited to continue all assessments.

Perhaps the most challenging questions facing clinical investigators planning a CNS clinical trial are:

1. What is the most appropriate type of participant to enroll in each phase of a trial program?
2. What would be the most accurate, sensitive, and reliable outcome measure for the chosen clinical target?

3. How is a clinical endpoint threshold selected to determine whether the therapeutic provides a meaningful clinical benefit to the experimental arm in comparison to an appropriate control group?

Each of these questions involves a number of theoretical and pragmatic considerations and an approximation is sometimes all that can be achieved in early trial phases. It is impossible to provide a detailed description of all the considerations for such decisions, but they can at least be mentioned. To limit errors in this discussion, I will primarily use examples from the disorder with which I am most familiar, SCI. Fortunately, the considerations influencing SCI trial design are similar to those for other CNS disorders, including stroke, traumatic brain injury (TBI), multiple sclerosis (MS), Parkinson's disease (PD), and dementia (including Alzheimer's disease).

Protocol Concern #1: What Is the Most Appropriate Type of Participant to Enroll in Each Phase of a Trial Program?

If we accept that every CNS disorder is heterogeneous in terms of symptoms, it stands to reason that those with the mildest or most severe forms of the disorder may be difficult participants to detect biological efficacy or functional benefits from an experimental treatment. When measuring outcome responses, such participants will generate statistical "ceiling and floor effects." In other words, taking all participants, regardless of severity and without stratification into more homogeneous subgroups, can result in a myriad of challenges including (1) risking the health and/or spontaneous recovery of people having a mild form of the disorder, (2) having the good responder effects in a mild form of the disorder statistically mask the poor treatment effects in severe forms of the disorder, and (3) having an outcome measure and clinical endpoint that does not provide the same degree of sensitivity and accuracy to detect a treatment effect across all severities of the disorder.

This is where having detailed knowledge of the characteristics and progress of the CNS disorder over time becomes valuable. Collecting large datasets that accurately track the presentation of the disorder's symptoms over time allows modeling of the natural history of the disorder. Such data can be most helpful to identify the neurological and functional trial endpoints a future control participant is likely to achieve with the current standard of care. This data can then be used to better estimate a clinical endpoint threshold that must be achieved to document an experimental treatment effect, as well as calculate the necessary sample size for a pivotal study phase (see below). If various trial measurement tools have been collected within such a natural history dataset, the responsiveness of these outcome instruments can also be evaluated for their sensitivity and accuracy to track change over the duration of the prospective trial program.

Such databases exist for almost every type of CNS disorder and they need to be utilized more often for modeling studies. One of the most robust and comprehensive

datasets for SCI is the European Multicenter study about Spinal Cord Injury (EMSCI). EMSCI is a consortium of approximately 20 European centers caring for people with SCI (http://www.emsci.org). For approximately 15 years, EMSCI has been prospectively tracking neurological and functional activities of people living with SCI at prescribed intervals during the first year after injury, specifically recording outcomes at time points <2 weeks and at 1, 3, 6, and 12 months after SCI. The EMSCI database has tracked outcomes from >3200 people (as of early 2015) who have survived and are living with various types of complete and incomplete traumatic SCI. This monumental EMSCI effort has enabled SCOPE and EMSCI researchers to investigate various scenarios with regard to future SCI trials and provide a more informed picture of more effective study protocols.

For example, EMSCI data have allowed investigation for the research value of clinical categories (A–E) normally used to describe SCI severity. Specifically, can these clinical categories predict future patient outcomes and will a change in category classification yield a sensitive and accurate tool to track and measure a treatment effect in a trial? It should be acknowledged that this classification system was only designed as a descriptive clinical shorthand and was never intended to be used as a trial measure. However, this adaptation of a descriptive neurological or rehabilitation classification to be a trial measurement instrument is an all too common occurrence for CNS disorders (Table 29.1).

Traditionally, the severity of SCI has been and continues to be clinically classified by a five-letter scale (A–E), first known as the Frankel scale and more recently named the AIS scale (for ASIA Impairment Scale where The *A* stands for American Spinal Injury Association; [20]). The AIS grades are a summary classification of the noninvasive neurological exam known as the International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI) [21]. With the AIS grade plus the

Table 29.1 American Spinal Injury Association (ASIA) Impairment *S*cale (AIS) modified from Hans Frankel

AIS grade	Type of spinal cord injury (SCI)	Defining characteristics of AIS grade
A	Complete injury	No motor or sensory function is preserved to S4–S5
B	Incomplete sensory, complete motor	Sensory, but not motor preserved to S4–S5 (with no motor function preserved more than three levels below motor level on either side of body)
C	Incomplete sensory and motor	Sensory function preserved to S4–S5 and either voluntary anal contraction or motor function more than three levels below motor level with less than half of the key muscle functions below neurological level of injury (NLI) scoring ≤3/5 on manual muscle test
D	Incomplete sensory and motor	Same sensory and motor function as described above for AIS C, but at least half of the key muscle functions below NLI have a muscle grade of ≥3/5
E	No injury (normal)	After prior deficits, sensory and motor functions have recovered to normal values at all spinal cord levels

segmental spinal level of SCI clinicians have a quick method to generally describe SCI (e.g., C4 AIS-A).

An AIS-A classification means there is no sensory or motor function preserved to the caudal limit of the sacral cord (S4–S5). Fundamentally, it means that most individuals have no preserved motor function, more than three segments below the initial motor level. AIS-B is the first incomplete SCI classification and means sensory function is preserved below the neurological level of injury (NLI), as far caudally as S4–S5 (i.e., anal sphincter), but there is no motor function, more than three segments below the motor level. AIS-C means there is sensory and motor function below the NLI, but half of the key muscles below the NLI have a muscle strength grade of <3 out of 5. AIS-D is the least neurologically impaired classification where more than half of the key muscles below the NLI have a muscle strength of ≥3 out of 5. Last but not least, an AIS-E means the person has normal sensory and motor function, even though they may have damage to their vertebrae.

As shown in several studies [14, 15, 17], AIS grade classifications are not sensitive or accurate in predicting the future prognosis of SCI or as a trial outcome measurement. Their biggest liability is their insensitivity to detect subtle change in function that may be clinically meaningful (e.g., a spinal segmental improvement in motor function; [12]). As a hypothetical example, presume we have a person who initially has a sensorimotor complete (AIS-A) SCI at the fifth cervical segment (C5). They can flex their elbow against gravity, but not against some resistance, and they have no other functionally useful upper extremity motor function below C5. The person is a tetraplegic and requires assistance to perform all ADLs including feeding, grooming, bathing, and dressing. Provision of an experimental treatment over 6 months stimulates functional motor recovery to C8 (but no further). The person is now independent with regard to all upper extremity ADLs and is functionally a paraplegic, but still categorized as AIS-A. Would such a therapy be judged clinically meaningful if it could be consistently achieved after cervical SCI? If you had set the primary trial endpoint to be a one or two grade improvement in AIS grade, such an individual would be judged to be a nonresponder or a treatment failure. Surprisingly, conversions of AIS grade have been recently used as a study endpoint in a cell transplant RCT [22] and an observational (early versus late decompression) surgical outcome study [23]. Now functionally meaningful spontaneous improvement in untreated SCI cases, with no change in AIS grade, is also common and underscores why such a trial endpoint has been generally abandoned [12, 14].

Admittedly, I have gone out of sequence and illustrated an insensitive (inappropriate) outcome measure, but this was done to broadly introduce different types of SCI participants for their suitability for possible recruitment to a study. A fundamental precept of any trial is “do no harm.” Consequently, most Phase I trials are conducted in uninjured healthy control subjects. However, this is unlikely to be ethical for an invasive CNS cell transplant. Thus, what SCI participants could ethically be recruited to such a study?

After evaluation of outcomes for people living with mid-thoracic sensorimotor complete SCI [11], it was noted that the segmental level for such a severe injury

rarely ascended two segmental levels spontaneously over the first year. Thus in the worst-case scenario for an adverse neurological event, it is very unlikely that any preserved upper extremity function would be harmed by such an invasive mid-thoracic surgical procedure. In short, the selection of thoracic level sensorimotor complete participants has been a very good model to confirm the safety of an invasive SCI therapeutic. To date, no participant with sensorimotor complete SCI has suffered any adverse event or significant change in neurological function as the result of an invasive cell transplant. Unfortunately, the considerable overlap in thoracic segmental motor function (trunk activity) means it has been very difficult to document a beneficial treatment effect in such study participants. To date, no consistent changes in lower extremity function have been observed after a treatment at a thoracic cord location. This may mean that most currently available experimental therapeutics will only exert a benefit within a few segments of the administration location.

Thus, thoracic sensorimotor complete subjects are not going to be good study candidates to recruit to a proper Phase II trial where the goal is detection of biological efficacy or functional improvement due to the provision of an experimental treatment. This has led investigators to recruit participants with cervical level SCI, whether the SCI is complete (AIS-A) or incomplete (AIS B–C). There are a number of outcome tools that can be applied to track neurological and functional changes after cervical SCI as the cervical cord mediates all sensory and motor function of the upper extremity (see Table 29.2).

But first, let us consider whether there are SCI participants that we might wish to not recruit to a clinical trial. As mentioned above, a person suffering a mild form of SCI (AIS-D) often retains some functional sensory and motor capacity. Furthermore, their recovery over the first year can be substantial. Regardless of the segmental level of SCI, if you measure the recovery of motor function (e.g., manual muscle test scores from 0 to 5) within key limb muscles, the recovery over the first 6 months can leave little measurement room to detect a beneficial treatment (ceiling effect). As a consequence, the sample size needed for such participants becomes impractical. In terms of safety, it is probably not wise to recruit an AIS-D participant to a drug or cell transplant SCI trial when the documented spontaneous recovery is so significant that a drug or cell treatment might unknowingly limit improvement.

In summary, we now know that the level and severity of SCI influences the inclusion and exclusion of specific types of SCI participants to different phases of a clinical trial program. Thoracic sensorimotor complete (AIS-A) participants are reasonable participants for recruitment to an invasive Phase I safety trial, but not for inclusion in subsequent Phase II or II efficacy studies (statistical floor effects). We know that mild forms of SCI (e.g., AIS-D) should never be included in an initial trial program for both safety concerns and for statistical ceiling effects. This leaves cervical AIS A-C SCI as high priority participants for recruitment.

Can similar distinctions be made for other CNS disorders? What criteria should be used to include or exclude stoke participants? Is it reasonable to recruit all stroke (cerebrovascular accident or CVA) victims, regardless of severity and location of

Table 29.2 Selected CNS disorders and outcome measurement tools associated with the disorders

CNS disorder	Associated outcome measurement tools
Dementia (Alzheimer's disease)	Mini-mental state examination (MMSE) Wechsler adult intelligence scale–revised Wechsler memory scale–revised Dementia rating scale Behavioral rating scale for geriatric patients (BGP) Alzheimer's Disease Assessment Scale–Cognitive Subscale Neuropsychological test battery Activities of daily living questionnaire Barthel index (BI)
Multiple sclerosis (MS)	Expanded disability status scale (EDSS) Box and blocks test Nine-hole peg test Berg balance scale Two-minute (or 6 min) walk test Twelve item MS walking scale Dynamic gait index Functional independence measure (FIM)
Parkinson's disease	Unified Parkinson's Disease Rating Scale (UPDRS, part III) and movement disorder society revision of UPDRS Berg balance scale Ten-meter walk test Two-minute (or 6 min) walk test Timed up and go Canadian occupational performance measure Craig handicap assessment
Spinal cord injury (SCI)	Int. standards for neurological classification of SCI (ISNCSCI and includes AIS grades) Spinal cord independence measure (SCIM) Functional independence measure (FIM) Spinal cord injury functional ambulation inventory (SCIFAI) Spinal cord injury functional ambulation profile (SCIFAP) Graded and redefined assessment of strength, sensibility, and prehension (GRASSP) Walking index for SCI (WISCI) Berg balance scale Two-minute (or 6 min) walk test Ten-meter walk test Timed up and go Canadian occupational performance measure Craig handicap assessment

(continued)

Table 29.2 (continued)

CNS disorder	Associated outcome measurement tools
Stroke (cerebrovascular accident or CVA)	Modified rankin scale (mRS) Barthel index (BI) NIH stroke scale (NIHSS) Fugl-Meyer assessment of motor performance Wolf motor function test Berg balance scale Two-minute (or 6 min) walk test Ten-meter walk test Timed up and go Functional independence measure (FIM) Stroke rehabilitation assessment of movement Rivermead motor assessment Stroke impact scale Canadian occupational performance measure Craig handicap assessment Action research arm test (ARAT) Sollerman hand function test Toronto rehabilitation institute hand function test
Traumatic brain injury (TBI)	Glasgow coma scale (GCS) and extended GCS Agitated behavior scale Montreal cognitive assessment Functional independence measure (FIM) Berg balance scale Two-minute (or 6 min) walk test Ten-meter walk test Timed up and go Canadian occupational performance measure SF-36 NeuroQOL Satisfaction with life scale Craig handicap assessment

CNS damage (e.g., cortical versus brainstem, unilateral versus bilateral, anterior versus middle versus posterior cerebral artery)? If not, how can we quickly and accurately stratify stroke participants for appropriate inclusion or exclusion? FAST (for *f*acial droop, *a*rm weakness, *s*peech difficulties, and *t*ime since onset) is a simple diagnostic evaluation for stroke, but it cannot clearly discriminate location or type of vascular insufficiency. Please note here: the acronym FAST is not the same as the FAST acronym used earlier in this chapter.

Magnetic resonance imaging can be a very good discriminator for the location of a stroke, as well as the type (ischemic versus hemorrhagic), but the time needed to complete an MRI assessment can exceed the time window suggested for the administration of some neuroprotective drugs [1]. Perhaps it is possible to prospectively declare that stroke participants can only be accurately stratified within a few days of their enrolment into a trial. If all participants are provided the same treatment, at least the treatment efficacy by stroke type can be better estimated and analyzed.

In terms of stroke severity, who is likely to be recruited to a trial? First, comatose individuals may be excluded due to severity and their poor prognosis. Likewise, mild forms of stroke (including transient ischemic attacks) often go unnoticed or are ignored (denial is common) by an individual until they have exceeded the window of opportunity for any neuroprotective treatment. Similar to SCI, stroke trials with a treatment window >6 h had approximately double the recruitment rates of trials that used treatment windows ≤6 h [24]. Equally important for the recruitment of stroke participants to longer-term rehabilitation studies is to screen for cognitive and language deficits so adequate comprehension of consent and rehabilitation activities can be appreciated [25].

The progressive degenerative CNS diseases, such as MS, PD, and the dementias, could be argued to be even more demanding than SCI and stroke in terms of discriminating different severities and types. The disorders are often slow in onset with little outward signs or symptoms at early stages and the syndromes often wane and wax (decrease and increase or remit and relapse). Categorization of MS and PD has become reasonably sophisticated based on decades of data collection and the ability to successfully image functional pathological damage [26]. The accurate classification of dementia is evolving, but probably some years away for confidently stratifying different types and severities of trial participants.

On the whole, careful consideration must be given to what type of study participant is appropriate for the therapeutic target of the trial. Taking all patients with a particular disorder is likely to create heterogeneous cohorts and lead to inclusive results. There is often a tenuous balance between recruitment of the appropriate type of trial participant and a need to recruit study subjects as quickly as possible. The most challenging situations in terms of heterogeneity are those studies where (1) the onset of the disorder cannot be determined, (2) the severity and the location of damage are difficult to define, or (3) the therapeutic must be administered within such a short window of opportunity that an accurate diagnosis cannot be performed.

Protocol Concern #2: What Would Be the Most Accurate, Sensitive, and Reliable Outcome Measure for the Chosen Clinical Target?

One example of an inappropriate selection of an outcome measure and clinical trial endpoint has been illustrated above (change in AIS grades for SCI). Unfortunately, it is not the only example. The general characteristics for a poor outcome measure are:

- It relies on the subjective evaluation of qualitative data (ordinal scales) that may not adequately cover the range of possible participant outcomes.
- It is insensitive (unresponsive) to detecting subtle changes over time.
- It has not been rigorously modeled for content or construct validity (e.g., Rasch analysis)

- It was adapted from a diagnostic tool used to describe a person's symptoms and investigators "hoped" it would also measure neurological or functional outcomes.
- It relies on a surrogate endpoint that has an unproven correlation to the disorder it purports to track (anatomical change does not always correlate with function).

Statisticians and clinical investigators have not always understood or appreciated each other's contributions; otherwise we might have had a higher success rate in CNS trials. This chapter is unlikely to be read by statisticians. Thus, for the clinical investigators, some goals and rules need to be mentioned:

- First and foremost—functional improvement "*rules*" as the only clinical endpoint that matters. Improved neurological status or more normal MR images might suggest the therapeutic is altering an appropriate target and you are encouraged to use such measures to track changes. However, improvement in function is all that matters at the end of a pivotal trial. If you cannot demonstrate improved and clinically meaningful function, you have failed!
- If you have not clearly understood and learned the natural history of recovery or deterioration associated with the disorder in question, you are doomed!
- If you cannot describe how you will conduct a pivotal Phase III trial before you finish a Phase II trial (some investors want this foresight before Phase I), you are likely to fail in your trial program!

Admittedly harsh statements, but surprisingly easy to defend and fortunately most experienced trialists have learned they must be able to positively respond with a plan to adequately address each of the above goals.

The most fundamental questions are:

- What are you going to measure?
- Why are you measuring these particular items?
- How can you be certain that the scoring options measure meaningful transitions in the chosen outcome item?
- How can you be certain that no uncontrolled variables will alter the accurate determination of this outcome?

Quantitative data are relatively easy to gather and summarize objectively. If a person has the capacity to stand and walk (with or without assistive devices), then measuring speed or distance is trivial, but how is it clinically meaningful? For example, a person with MS takes one medication and can now walk at a reasonable pace, but has a large degree of spasticity. They then take an appropriate muscle relaxant (antispasmodic drug) and now walk with less painful muscle spasms, but at a slower pace. Which outcome is more clinically meaningful? The perspective of the participant may only resolve such distinctions and this is why PROs have become an essential part of trial measurement.

Although we can noninvasively measure brain activity using a number of quantitative and semi-quantitative tools (EEG, MRI, fMRI, and PET), we can only grossly correlate a quantitative measure of brain activity with a specific CNS function or behavioral activity. Since function is the desired clinical endpoint, investigators try

to capture as many of a person's functional capacities after CNS damage using descriptive qualitative (ordinal) scales. But, this generates a new set of concerns. There can be doubt as to how sensitive and responsive these ordinal scales are and whether each scoring option within the scale's range is linear and can describe a meaningful change (functional transition)? Perhaps an illustration would help.

A popular outcome measurement scale for the perception of pain is the visual analog scale (VAS). Generally speaking a 10 cm horizontal line is used (with scoring intervals at each mm to generate a scale with a scoring range from 0 to 100). Participants are asked to place a mark on the line at a point representing the severity of their pain with the left hand anchor (0) equal to "no pain" and the right hand anchor (100) denoting "pain as bad as it could be.". Please note, researchers can also ask for a verbal report or use a numeric rating scale (NRS) with interval values from 0 to 10 (it is essentially the same pain scale). Most importantly, precision is a function of the accuracy of a measurement scale. False precision can arise when more response categories (scoring options) are created than are actually needed to describe a functional or meaningful transition (e.g., being able to precisely time to 0.001 s on a handheld stop watch is of no value when a human cannot reliably and repeatedly function within that timescale). Does a 100-point scale make it easier or harder for a person to accurately assess the level of their pain than a 10-point or a 5-point scale. More importantly, does it provide increased precision when asking a person to compare their current pain to the pain they experienced at some previous assessment (a measure of scale responsiveness)?

Thus the VAS is a subjectively scored ordinal scale; nevertheless, investigators often treat the VAS as a continuous scale (each interval measuring an equal increment in perceived pain intensity) and presume it is acceptable to then use parametric statistics [27]. However, just because some researchers assume the scale is linear in nature, does not necessarily mean this assumption is correct.

It has been shown that the VAS does not behave linearly for patients with all levels of pain [28]. Measuring change scores along the VAS scale will be invalid unless the intervals between each scoring option are of equal value and selection of a score can be further compromised by participant errors in estimation [27]. Rasch analysis (an advanced form of clinimetric or psychometric analysis) allows an investigation of person fit, which examines if people use the scale as expected, given the change in item difficulty along the scale and their total score or change score along the scale. In traditional psychometric testing this is not examined; indeed, the assumption is made that people respond to items in the way investigators intended (or hoped). Using Rasch analysis, it has been noted that the VAS only behaves in a linear fashion in the middle of the scale, but in a nonlinear manner at the lower and upper ends of the VAS. Rasch modeling clearly demonstrated that VAS is not a linear scale and parametric statistics should not be used (e.g., mean, standard deviation, parametric methods for calculating sample size). VAS may be valid as a measurement of pain at one point in time for an individual, but the lack of scale linearity means change scores (over time) using VAS are meaningless [27].

The findings by Kersten and colleagues [27] highlight the value of logistic clinimetric analysis, such as Rasch modeling, to test the validity for converting the available

"raw" ordinal data to more appropriate interval values, prior to interpretation. More importantly, the findings raise serious issues for researchers using raw VAS scores for pain and the interpretations of their results. If a raw VAS scores are used as a primary outcome measure, it must either be subjected to nonparametric statistics, or transformed by Rasch analysis into an interval scale where parametric statistics can be used. Given that so many of the CNS measurement scales are ordinal should cause everyone to seriously reflect on the accuracy and sensitivity of their measurements and consider how they can most accurately validate their metrics.

In summary, there are numerous measurement scales (see Table 29.2) and several of these measurements have been used for a number of different CNS disorders. Almost all the measures have been examined for simple psychometric (clinimetric) properties such as inter-rater reliability, test–retest reliability by the same examiner, content validity (refers to how accurately an assessment or measurement tool subjectively represents all aspects of the intended scale domain), and construct validity (refers to the degree a scale measures what it purports to measure). Construct validity is sometimes substituted by concurrent validity where a new scale is validated by the fact it correlates well with previously used scales; of course, they could all be measuring the wrong items! As can probably be surmised, there is a sufficient amount of doubt associated with any of these validation attempts when you do not know what will denote an improvement in a CNS disorder. While validity is relative, reliability can be objectively described and why there are more publications on reliability, which is just a measure of the consistency of scoring. A delta change from baseline is often the most important metric for a clinical trial, yet few of the available ordinal scales have been examined for their responsiveness over time and whether the scale tracks a change from an easily accomplished task to increasingly more difficult functional activity task.

Thus the classical psychometric examinations are slowly giving way to more advanced logistical statistics (e.g., Rasch modeling), which can rigorously establish whether a scale provides a progression along a continuum from easily accomplished to more difficult to achieve. If there is any disorder (i.e., the item or scoring option for that item is not working as intended to measure increasing difficulty), Rasch algorithms will identify the item or response scoring of an item that needs to be improved. Disorder is created when the probability of a response score cannot be reliably selected to describe a meaningful transition in the item. In such an instance, the outcome assessor will not be able to consistently select the appropriate score to describe that item (function). Rescoring conservatively so as not to shorten the overall range of scoring options can remove disorder, but this requires clinical experience with the item and its value to the assessment of meaningful behaviors. A number of questions should be raised when ordinal scale has an overall scoring range that "nicely" fits a metric of 0–10 or 0–100. Until proven otherwise, there will always be concern when the interval between each successive score is not the same magnitude as it is with continuous quantitative scales (e.g., time and distance).

In brief, there is no lack of psychometric or clinimetric studies reporting content or construct validity and intra-rater or inter-rater reliability, as these are relatively easy assessments. The more important metric of scale responsiveness to sensitively

and accurately track and measure a change in function from baseline appears to be the forgotten psychometric property. Rigorous responsiveness testing, such as Rasch modeling, has only appeared recently. Unfortunately, most of the present Rasch reports (e.g., [27]) often conclude with a statement that the scale is not linear and has an unacceptable degree of disorder in the scoring of items. No effort is made to try to rescore clinically important items (the third point from the above list) to make the scale linear and validate that the scores show effective transitions in the items across the overall range (from the easiest to the hardest item for a participant to reacquire). This will only happen when statisticians work alongside clinical investigators to nominate reasonable functional transitions. A collaboration of this type is in the final stages of completion for SCI by SCOPE.

Turning to the conceptual questions "what and why are you going to use as an outcome measure for a CNS trial" we are confronted with the need to construct a hypothesis that makes sense for a therapeutic directed to alter CNS tissue activity. Measurement of changes in sensation is relatively straightforward and important for sensory CNS disorders, but many of the most prevalent CNS disorders involve an impairment of motor behaviors. All ADLs rely on motor functions that include both involuntary components (e.g., reflexive and autonomic) and voluntary actions (intended and directed). Thus, should we try to measure all bodily functions or do we focus on volitional performance of the appropriate cognitive and physical tasks contributing to greater independence in basic ADLs? Measuring activities dependent on volitional motor performance is an outcome that is less encumbered by the uncontrolled variables intrinsic to autonomic nervous system activities. It has been a focus of SCI trials and intuitively makes sense. Most CNS disorders (TBI, stroke, MS, PD, dementia, etc.) also have impairment of volitional motor performance.

Reviewing several scales used to measure a change over the duration of a CNS trial indicates that several components are related to assessing volitional motor performance (www.rehabmeasures.org; www.neuropt.org/professional-resources/neurology-section-outcome-measures-recommendations). As Table 29.2 shows there is an abundance of instruments (scales) directed to measuring various aspects of a number of CNS disorders. They include measures that assess outcomes within the structure/function, activity, and participation domains of the International Classification of Functioning, Disability and Health (ICF) of the World Health Organization (www.who.int/classifications/icf/en/). Many of these outcome measures are PROs and include perceptions of participation within the community and QOL. Apologies if your favorite outcome measurement tool is not listed in Table 29.2, but each CNS disorder has at least 50 different assessments to which it has been linked. These staggering numbers emphasize two fundamental issues: (1) we do not need any new outcome measurement tools, and (2) we need more rigorous responsiveness testing of the current outcome instruments.

Stroke has predominantly utilized three outcome measurement scales in clinical studies, the NIH stroke scale (NIHSS), the Barthel index (BI), and the modified Rankin scale (mRS). The ideal scale would be quick and easy to complete, valid, reliable, and responsive to detecting meaningful clinical (functional) change. Since several of these conditions are at odds with each other, it is not surprising that there

Table 29.3 Modified Rankin scale: a simplified outcome measurement scale used in stroke trials

Score	Defining characteristics for this score
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability, unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent, and requiring constant nursing care and attention
6	Dead

is no ideal, universally accepted, stroke outcome scale. The NIHSS appears to satisfy content validity, reliability across observers, although some items are considered redundant (nonlinear or disordered) and it may not be as sensitive for non-dominant hemisphere stroke syndromes [29]. The BI is more commonly used in rehabilitation settings as it tracks ADLs, but the major concern with the BI is the limited responsiveness to change (floor and ceiling effects are common). The major concern with the mRS is the limited number of scores (Table 29.3) which may make the mRS less sensitive (responsive) to detect a subtle but meaningful change (a type II error, where a beneficial treatment effect is missed). The mRS can be likened to the AIS grades for SCI (described above) and may have the same shortcomings. Finally, does a single point change always describe a clinically meaningful change (see below)?

The fourth point from the list above emphasized the possibility that variable beyond your control as a clinical investigator can alter clinical trial results. There are always concerns about how to control for independent variables and how they could alter the accurate interpretation of trial outcomes. One example is the amount of rehabilitation a person might be receiving during a study investigating a novel drug, cell transplant procedure, or implanted device. The number of independent variables increases with each successive ICF domain (from structure/function to activity to participation outcomes). In brief, there is not much that can be done to eliminate an independent variable. You should not attempt to dictate or restrict the lifestyle of trial participants. However, you can track or conduct surveys on the potential independent variables that you cannot control. Beyond rehabilitation, these might include an unrecognized preexisting health condition or comorbidities, compensatory or adaptive behaviors, psychological well-being, motivation, community/family support, and financial resources. For example, it has been reported that preexisting dementia could contribute to the cognitive deficits observed after stroke [30]. This might skew the results sufficiently for a drug to be judged as an ineffective therapeutic. The more a trial endpoint relies on a descriptive PRO, the greater concern there is for the influence of independent variables on trial outcomes.

Protocol Concern #3: How Is a Clinical Endpoint Threshold Selected to Determine Whether the Therapeutic Provides a Meaningful Clinical Benefit to the Experimental Arm in Comparison to an Appropriate Control Group?

Unless, there is a well-established clinical trial history, as in PD [31], selecting a clinical endpoint can be a most difficult decision. Currently, investigators recognize the heterogeneity within any individual CNS disorder and the variability in the rates and extent of recovery or deterioration across differing severities and/or locations of the CNS damage. It is widely accepted that there are also an increasing number of uncontrolled independent variables that can influence the accurate interpretation of trial findings as outcomes move across the ICF domains. Thus, each CNS disorder faces ambiguity until a novel therapeutic is validated as a new ("gold") standard of treatment, which can then be used as a comparator for future studies [19].

As mentioned above, a commonly used outcome measurement scale for stroke is the mRS (Table 29.3). The scale has only seven options for scoring (0–6), with 0 used for a normal outcome (no symptoms) and 6 used for "dead." It is a relatively simple outcome instrument and favored for large multicenter trials. There have been many systematic reviews detailing the standard psychometrics of the tool (construct validity and inter-rater reliability), but little study of the responsiveness and equal sensitivity (linearity) of the measure across all the scoring options from 0 to 6. What scoring change would constitute a minimal clinically important difference (MCID) could also be debated. A one-point change for a participant moving from a 4 (moderately severe) to 3 (moderate) or from a 3 to 2 (slight disability) might be functionally important, whereas a similar change from a 6 (dead) to 5 (severe disability) may not be seen as meaningful or a desired outcome by most patients, their family, or society as a whole. Thus, where do we set the threshold for a treatment effect? Only prospective modeling of spontaneous recovery patterns will enable a threshold to be set with some justification and confidence and then only for a homogeneous study population.

Modeling the available natural history data can be useful as it allows an investigator to establish the magnitude and rate of recovery or deterioration for potential control participants, over a given period of time, equivalent to the study duration. A statistical estimate of the minimal detectable difference (MDD) can then be made and this value, along with the magnitude of the probable spontaneous recovery, must be exceeded to set a valid clinical endpoint threshold for any experimental trial participants [16]. The MDD statistic describes the smallest real change in the specified outcome, beyond measurement error, and it should not be confused with the minimum threshold for demonstrating the MCID. Many investigators believe that MCID can be derived statistically. This is a fallacy, and for a more complete discussion of the history of MCID and how it can be estimated, please read the review by Wu and colleagues [16] as it covers the conceptual framework for MCID. In brief, it is not difficult to make appropriate endpoint threshold calculations, but it is time

consuming. It should be remembered that accurate calculations for the effect size of a treatment are necessary for estimates of sample size (power calculations).

MCID, as originally introduced by Jaeschke and colleagues [32], was defined as "the smallest difference in score, within the domain of interest, which patients perceive as beneficial and which would mandate, in the absence of troublesome side effects and excessive costs, a change in the patient's management." By this definition, MCID is only defined by the minimal amount of change (treatment effect) that is important to people living with the disorder; the experienced wisdom of clinical investigators is excluded. MCID is an attractive and useful PRO concept, especially for chronic CNS studies where people have developed an awareness of what improvements provide personal benefits, such as increased independence in ADLs, improved QoL, and reduced chronic pain. At this point, it is not clear how MCIDs can be emphatically defined for each outcome measurement, nor it is clear how an acute trial MCID might differ from an MCID for a chronic study.

MCID emphasizes the primacy of the perspective of the person living with a disorder, which hopefully correlates with that of the clinician or caregiver. In fact, investigators should take an active role in defining MCID where and when participants might not be sufficiently informed to make an adequate determination independently. Best practices in clinical care increasingly emphasize shared decision making in determining treatment goals [8]. Although the MCID and PRO concept appears to be easily understood by the trial investigators [33], it does involve the inherent risk of a trial participant setting an unrealistic trial endpoint, whether it is too high or too low [34]. At this time, the best strategy that can be offered for determining a CNS therapeutic benefit must rely on the correlation between a structure/function measure and a relevant activity outcome, as well as statistically significant differences between experimental and control study cohorts.

Conclusions

I have not covered the history of CNS clinical trials, but it is safe to summarize that past translational efforts were naive because (1) they either did not understand the necessary elements for validating and developing an experimental treatment at the preclinical level and/or (2) they hoped for an unreasonably large treatment effect to get them past any inherent clinical trial problems associated with a heterogeneous study population or an inappropriate trial outcome measurement. As outlined above, experience, errors, and careful reflection have taught us what fundamental translational elements must be satisfied to move clinical trials forward effectively and efficiently. Shortcuts and stubborn refusal to learn from past lessons usually lead to failure; hopefully we are becoming wiser.

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Chapter 30
Conclusion

Mark H. Tuszynski

Keywords Transcriptome • Proteome • Gene therapy • Antisense therapy • Alzheimer's disease • Parkinson's disease • Autism • ALS

This book has covered translational programs in many neurological arenas: neurodegenerative disorders, trauma, stroke, epilepsy, multiple sclerosis, genetic disorders, pain, and cancer. It has addressed techniques central to many translational efforts: genomics, gene therapy, antisense therapy, stem cell therapy, drug discovery/development, bioengineering, and rehabilitation. We have focused on efforts currently in late-stage clinical development or in actual clinical trials; many additional research programs' efforts also strive to generate sufficiently compelling data to merit human translation.

The topics we have covered focus primarily on translational efforts that have adopted novel technologies. We thereby provide less perspective on traditional drug design and development, but the latter efforts in many cases also exploit genomics, proteomics, and other new fields to identify and translate the most promising drug candidates.

None of the topics covered in this book have yet yielded positive findings from Phase 3 clinical trials, with subsequent regulatory approval. Hopefully, the next edition of this book will report such success, but today it is too soon for that. As implied in several preceding chapters, the jump from nonhuman animal models to human clinical trials is enormous. As good scientists in the laboratory, we strive in our preclinical models to test a central hypothesis, while constraining uncontrolled variables: this effort attempts to minimize background noise and allow the potential "signal" of a candidate therapy to become recognizable above the background variability. Yet, when we subsequently enter the arena of human clinical trials, this effort to control uncontrolled variables is daunting. A large number of uncontrolled variables enter into play in human clinical trials, unlike our preclinical animal experimentation, converting the background noise of variability into a roar (Table 30.1).

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Table 30.1 The noise gap between preclinical and human studies

Preclinical animal studies	Human clinical trials
Animals of same strain, often inbred	Humans have extensive genetic heterogeneity
Subjects of same age	Subject age variable
Often one gender studied	Both genders usually studied
Uniform health background	Concomitant and variable diseases: hypertension, diabetes, immune disorders
No exposure to nonstudy drugs	Numerous other drugs: anti-inflammatories, antibiotics, antihypertensives, etc.; many of these untracked
Same lesion type, disease model	Different mechanism, severity, time to treatment, and duration of disease
Same experimenter administering lesion	Cause, etiology can vary considerably
Same rater assessing outcome	Numerous raters assessing outcomes at many different sites, often applying or interpreting assessment tools differently
Controlled, consistent diet	Highly variable diet and diet-related confounds including vitamin deficiency, obesity, others
Consistent "lifestyle" and activity	Highly variable physical activity and lifestyle

While every effort is made to control the "noise" of the clinical trial, this is invariably far more complex than the preclinical experiments that yield our translational candidates. As a consequence, an experimental manipulation that yields a consistently positive signal in preclinical studies may simply experience a washout of that effect by the "noise" of the human clinical trial. Indeed, folklore in the clinical trials arena suggests that 95 % of human clinical trials fail (some drug development experts believe that this number is an underestimate; few argue that it is an overestimate).

In this context, it may not be that surprising that candidate translational neuroscience programs need some time to yield success in clinical trials. As a translational program progresses into initial human trials, we learn a great deal from these early clinical efforts, even failed efforts, that can be used to shape subsequent clinical trials and result in a better ability to eventually detect benefit. For example, as cited in Chap. 3, human brain gene therapy trials are only now acquiring tools that are more likely to allow accurate gene targeting and "dosing" to affected brain regions. It is equally likely that early stem cell clinical trials will experience a learning curve regarding *how* to graft cells, *where* to place them, and how to optimize the nature and duration of immunosuppression. These efforts take time.

One can argue that a means of dealing with the "95 % problem" of human clinical trial failure is to test a candidate therapy in the most relevant animal model of a particular human disease that could optimize positive clinical predictive value. While testing in the most relevant animal model need not be part of initial screening efforts to identify a candidate therapy, common sense suggests that it becomes

advisable to test a candidate therapy in the most relevant model to human disease as part of the decision process to move a candidate therapy to humans.

What is a clinically relevant model? "Relevance" to human disease reflects similarities among nonhumans and humans in disease mechanism, severity of injury, timing of initiation of therapy, duration of therapy, outcome assessment (using similar rating scales in humans and experimental animals), and other factors. For example, some therapies have progressed to clinical trials in chronic spinal cord injury without actual assessment in animal with chronic injuries; this paradigm entails high risk. Other human trials have gone forward when candidate therapies have only been tested as pretreatments, before disease onset; this too appears to make little sense. Recently, some candidate therapies have been proposed for human trials based on only in vitro data, without any in vivo testing at all. These approaches increase the risk of sustaining or expanding the "95 % problem."

Researchers of various neurological disorders face distinct challenges in generating animal models that are relevant to their human disease. In spinal cord injury, one can readily generate a severe compression injury in experimental animals that mimics the severity of human trauma, and one can administer therapies in a way that would closely mimic clinical protocols in a human clinical trial. Surprisingly, however, few translational trials in spinal cord injury have actually tested their candidate therapies in severe lesion models, however. In Alzheimer's disease research, no animal model has yet consistently and reproducibly recapitulated the nature of the human disease; this is particularly challenging in Alzheimer's disease because a principal risk factor for disease is advanced (human) age. In AD, therefore, a decision to advance a therapy to the clinic may rest upon positive signals generated across several different yet imperfect correlative models of the disease. Each disease is different.

Statistical analyses in preclinical studies merit special mention when discussing the identification of optimal candidate therapies for clinical translation. Clinical trials have a history of particular strength in this arena, and general statistical practices in the pharma and biotech industries often provide a model for improving methods used in academic laboratories. The most reliable data are collected and analyzed in a blinded manner, using sufficient sample sizes to generate meaningful conclusions. In particular, it is standard practice in clinical trials to *prospectively* plan the exact statistical methods that will be used to judge outcome of a clinical trial. Moreover, the precise outcome measures that will be given consideration are listed and weighted for impact (e.g., "primary outcome measure"). Furthermore, the manner in which *missing* data points at the end of the study will be managed is also established prior to the initiation of the clinical trial. These standards are very rarely applied in preclinical animal studies, and academic laboratories are probably the least likely to adopt them. There are various reasons for this: academic laboratories conduct earliest stage discovery research, and many of these studies seek to identify any signal of a possible biological effect. This can funnel down from a broad array of potential biological candidates to the most robust candidates, although less rigorous methods also carry a high risk of identifying false positives. Nonetheless, "exploratory" anal-

Table 30.2 Identifying optimal candidates for translation

Objective	Rationale
Test in most clinically relevant animal model	Enhances positive predictive value in clinical trial
Robust effect in preclinical testing	Addresses high "noise" in human trials
Identified mechanism of action	Allows drug/therapy optimization
Independent replication	Suggests consistency and robustness of effect
Large animal testing	Ensures scalability of drug/therapy, allows development of methods for delivery, supports dosing, confirms effect
Statistical analysis	Prospective, rigorous, blinded data analysis plan

yses of early-stage experiments can be reasonable when experimenters are aware of the caveats of this approach. However, once a candidate translational therapy has been identified from a series of less rigorous, early-stage experiments, an ultimate set of preclinical experiments should be designed using the best animal model and an appropriate, prospective plan for statistical analysis.

From the preceding discussion, an approach to identifying candidate therapies for translation can be offered (see also Chap. 29) (Table 30.2).

In summary, we live in an era of unprecedented potential for translational neuroscience. Rapid advances in our understanding of disease mechanism based on genomics, proteomics, cell biology, bioinformatics, imaging, and engineering have generated a new array of potential tools for treating human disease. This edition of *Translational Neuroscience* reveals the potential of many of these approaches; we hope that future editions will report their initial successes and the transformation of these successes into the broad availability of treatments for diverse neurological disease indications.

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